Studies on the molecular regulation of nutrient supplementation in carnivorous *Nepenthes*

Dissertation

To fulfill the
requirements for the degree

„doctor rerum naturalium“ (Dr. rer. nat.)

Submitted to the council of the faculty of biology and pharmacy of the Friedrich-Schiller- University Jena

by
Dipl.-Biochem. Ayufu Yilamujiang
born on 17.09.1983 in Kashgar

Referees:

Referee 1: PD Dr. Axel Mithöfer

Referee 2: Prof. Dr. Severin Sasso

External Referee: Prof. Dr. Irene Lichtscheidl

Date of defense: 15.08.2017

Table of Contents

Summary 1

Zusammenfassung 3

1. General introduction 5

1.1 Carnivorous lifestyle in plants 5

1.2 Protein composition of *Nepenthes* pitcher fluid 7

1.3 Plant pathogenesis related proteins 9

1.4 Regulation of carnivory-related processes in carnivorous plants 10

1.5 Coprophagous features in *Nepenthes* 12

1.6 Urea and urease in plant 14

1.7 Aim of the thesis 16

2. Manuscript overview 17

2.1 Manuscript I 17

2.2 Manuscript II 18

2.3 Manuscript III 19

3. Manuscripts 21

3.1 Manuscript I 21

3.2 Manuscript II 37

3.3 Manuscript III 45

4. Unpublished results 69

5. General discussion 74

6. References 82

7. Curriculum Vitae 87

8. Acknowledgement 90

9. Eigenständigkeitserklärung 92

Summary

All organisms on earth are forced by environmental challenges for millions of years to either adapt, or escape from inhospitable habitats. Being sessile organisms, adaptation is the only alternative for plants. High plasticity in plant metabolism, biochemical and signaling pathways, and morphological variations promote the performance of plants under stressful environmental conditions. Plants also successfully co-exist with other organisms such as microbes or insects. As an adaptation mechanism for nutrient limited habitats and soil, carnivory has developed from plant-insect interactions. Most terrestrial carnivorous plants such as *Nepenthes*, *Dionaea* and *Drosera* use diverse trapping organs; however they all specialize in insect prey digestion to have access to prey-derived nutrients. Carnivorous plant of the genus *Nepenthes* with over 120 species uses so-called pitchers, which are metamorphosed leaf organs with digestive fluid inside. Identified protein compositions of the digestive fluid are categorized in the families of pathogenesis related (PR) proteins. Only few species of *Nepenthes* developed mutualistic relationships with mammals for nitrogen supplementation allowing further coprophagous specialization. Here, we show the following results: Insect prey as well as chitin is able to first induce jasmonate phytohormones, which in turn can induce genes of digestive enzymes such as a chitinase and a protease, nepenthesin. For the latter, prey- and chitin-induced activity was also detected in the pitcher fluid. Moreover, external jasmonic acid application was sufficient to induce proteolytic activity of the fluid, indicating the important role of jasmonate signaling in prey-digestion related gene expression. Putative *cis*-acting regulatory elements are observed on the promoter regions of selected PR genes of the digestive fluid proteins. Some of those potential promoter elements are known to be involved in plant defense regulations. Apart from prey-derived nutrient acquisition, *Nepenthes* was also capable to metabolize ^{15}N-enriched urea and following which the nitrogen is distributed within the

plant. Exactly this ability of the plant, which is facilitated by the enzyme urease, allowed further specialization, namely coprophagy. In such case, the plants benefit from animal defecation. Urease genes from coprophagous and carnivorous *Nepenthes* species were cloned and functionally expressed. A comprehensive phylogenetic analysis for eukaryotic ureases, including *Nepenthes* and five other carnivorous plants' taxa, identified them as canonical ureases and reflects the plant phylogeny. Overall, the results of my thesis demonstrate the ability of *Nepenthes* plants to shape existing pathways in favor of carnivory or even coprophagy. It, furthermore, confirms the hypothesis that carnivory has evolved from plant defense strategies and that this lifestyle seems to have evolved convergently in the different taxa of carnivorous plants.

Zusammenfassung

Alle Organismen auf der Erde sind seit Millionen von Jahren durch ihre direkte Umwelt gezwungen, sich entweder an unvorteilhafte Lebensräume zu adaptieren oder sie zu verlassen. Pflanzen als sessile Organismen haben keine andere Wahl als sich zu anzupassen. Der Erfolg bei der Adaptation von Pflanzen wird dadurch gefördert, dass sie eine hohe metabolische, biochemische und auch das Signalisieren betreffende Plastizität besitzen. Die Pflanzen sind nicht nur in der Lage zu adaptieren, sondern auch mit anderen Organismen wie zum Beispiel Mikroben oder Insekten zusammen zu leben. Als Anpassung an nährstoffarme Umweltbedingungen und Böden ist eine spezielle Variante der Pflanzen-Insekten Interaktion entstanden, die pflanzliche Karnivore. Die terrestrischen fleischfressenden Pflanzen wie zum Beispiel *Nepenthes*, *Dionaea* und *Drosera* besitzen unterschiedliche Fangmechanismen, die durch verschiedenste Fallentypen realisiert werden. Alle diese Pflanzen sind darauf spezialisiert, Insekten zu fangen und zu verdauen und profitieren von den so gewonnenen Nährstoffen. Die mit über 120 fleischfressenden Arten große Gattung *Nepenthe*s, besitzt als Falle sogenannte Kannen, die durch Metamorphose des Blattes entstanden sind und mit Verdauungsflüssigkeit gefüllt sind. Die identifizierten Proteine, die in der Kannenflüssigkeit vorkommen, können den Familien der „*pathogenesis related*“ (PR) Proteine zugeordnet werden. Nur wenige Arten von *Nepenthes* haben ein mutualistisches Zusammenleben mit Säugetieren entwickelt, wo sie die Ausscheidungen der Tiere als Nährstoffquelle nutzen. Die folgenden Ergebnisse werden in der vorliegenden Arbeit demonstriert. Die Fütterung der Kannen, sowohl mit Insekten als auch mit Chitin, induzierten die endogenen Phytohormon Konzentrationen und die Gene, welche die für die Verdauung verantwortlichen hydrolytische Enzyme kodieren, wie zum Beispiel eine Chitinase und die Protease Nepenthesin. Es wurde weiter gezeigt, dass die hydrolytische Aktivität der Kannenflüssigkeit durch Blattmaterial, Insekten und auch reines Chitin induziert werden kann. Außerdem konnte allein die externe Zugabe von Jasmonsäure die

hydrolytische Aktivität der Flüssigkeit erhöhen. Dies bedeutet, dass Jasmonsäure-abhängige Signalweg eine Rolle bei der Etablierung von hydrolytischen Aktivitäten in *Nepenthes* spielt. In den Promotor-Regionen von PR Genen in *Nepenthes* können *cis*-aktivierbare, regulatorische Promotorelementen vorhergesagt werden, die in der Pflanzenabwehr eine Rolle spielen. Über die Insekten-verdauenden Fähigkeiten hinaus haben die Kannenpflanzen gezeigt, dass exogener, ^{15}N-angereicherter Harnstoff von der Pflanze metabolisiert und der freigesetzte Sticksoff in der Pflanze verteilt werden kann. Diese Fähigkeit, basierend auf der Aktivität einer Urease, hat dazu beigetragen, dass die Kannenpflanzen koprophage Eigenschaften entwickelt haben; sie können direkt von den Ausscheidungen der Tiere profitieren. Urease Gene aus zwei *Nepenthes* Arten, eine karnivore und eine koprophage, wurden kloniert und heterolog exprimiert. Ein umfangreicher phylogenetischer Stammbaum für Urease von eukaryotischen Organismen (Pflanzen, Pilze), der erstmals Ureasen aus sieben verschiedenen fleischfressenden Pflanzen enthält wurde des Weiteren erstellt. Die Analyse hat für die Ureasen der karnivoren Pflanzen kanonische Eigenschaften demonstriert. Die Ergebnissen der Arbeit haben gezeigt, dass *Nepenthes* Pflanzen in der Lage sind, vorher schon existierende Signalwege zugunsten der Karnivore, sogar für koprophage Eigenschaften zu nutzen. Außerdem kann die Hypothese bestätigt werden, dass die Karnivore aus der pflanzlichen Abwehr hervorgeht und in konvergenten Entwicklungen in verschiedenen Taxa der karnivoren Pflanzen entstanden ist.

1. General introduction

1.1 Carnivorous lifestyle in plants

Plants are unable to escape from unfavorable environmental habitats. Instead they have evolved strategies to adapt to harsh conditions, fend off pathogens, outgrow competitors, and reach sources of nutrients (Wolters and Jürgens, 2009). As an adaptation mechanism to survive the limited nutrient acquisition in natural inhospitable habitats, some plants developed a carnivorous lifestyle (Juniper *et al.*, 1989).

Since the time of Charles Darwin, carnivorous plants have attracted scientific interest for their amazing performance on insect prey digestion-based living. Darwin published a book about carnivorous (insectivorous) plants (Darwin, 1875) describing pioneer studies on this interesting topic, which encouraged scientists up to now to study the molecular mechanisms of carnivorous plants; however, nowadays by applying modern technologies.

Carnivorous plants are able to supplement their nutrient demand with their unique features of prey capture and digestion. They catch arthropod prey, mostly insects, with the help of modified leaves, which are fundamental to fulfil extraordinary features associated with plant carnivory (Mithöfer, 2011). Carnivorous plants have five basic trapping mechanisms: pitfall trap, adhesive trap, snap trap, corkscrew trap, and suction trap (Juniper *et al.*, 1989). Trapping-organ based prey capturing strategies vary in different species, actually even between genera (Juniper *et al.*, 1989). Characteristics of the trapping organs are unique in carnivorous plants according to the belonging of more than 580 species to five orders and 20 genera (Givnish, 2015). Basically, hydrolytic enzymes secreted from special glands perform prey digestion and hence supply the plants with prey-derived nutrients; mainly nitrogen and phosphorus (For detail see 1.2).

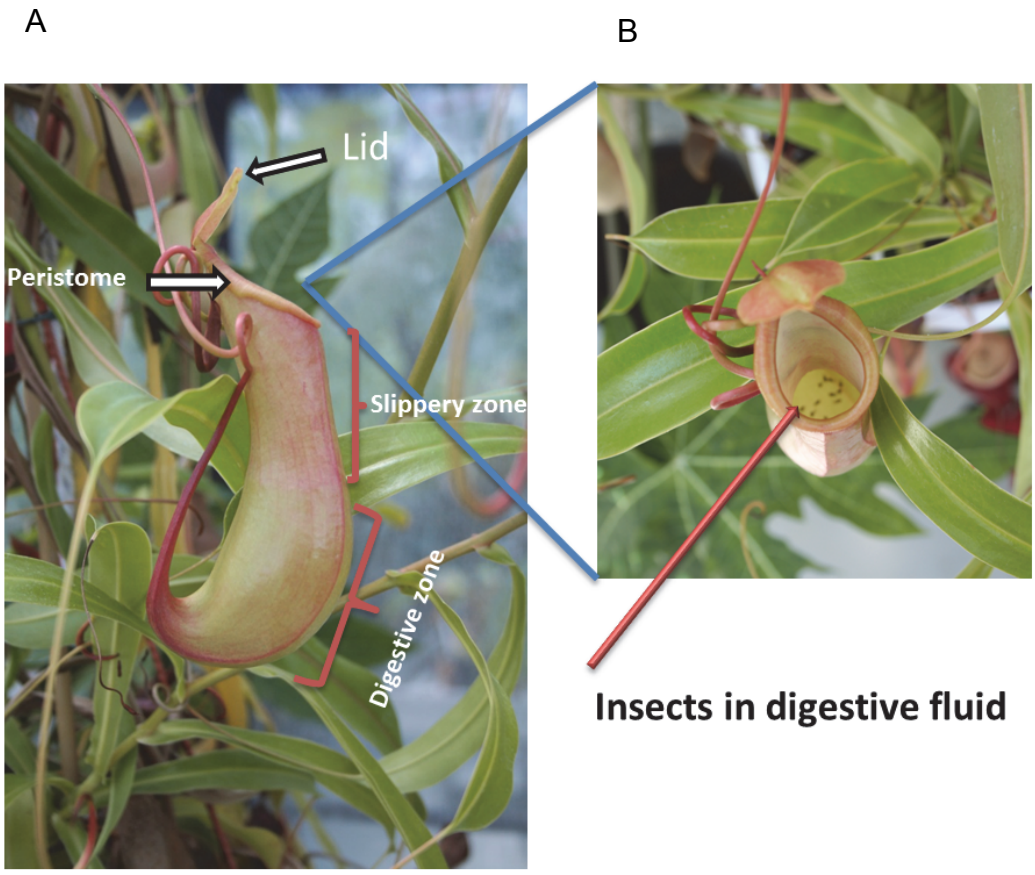


Figure 1. (A) Shown is the carnivorous plant *Nepenthes alata* with the metamorphosed leaf organ, pitcher. Pitchers consist of a digestive zone, slippery zone, peristome and lid. Pitchers contain a digestive fluid inside the digestive zone. (B) Insect prey present in the digestive fluid.

Within the order of Caryophyllales, Nepenthaceae and Droseraceae are the most investigated and prominent carnivorous plant families. In the latter family, an adhesive trap is used by *Drosera spp.*, whereas a snap trap is unique for *Dionaea muscipula* as the only species of its monotypic genus. Within Nepenthaceae, only the genus *Nepenthes,* with more than 120 species is native to South East Asia. Its highest diversity is found on Borneo (Juniper *et al.*, 1989). For prey capture, *Nepenthes* plants use a metamorphosed leaf organ, the pitcher (Figure 1). Particularly three zones are characteristic for the pitcher: first the peristome at the top of the pitcher

that is involved in attracting and trapping the prey; second a slippery waxy zone inside the pitcher, which is involved in trapping and preventing prey escape; and third a digestive zone at the inner bottom part of the pitcher that is covered with bifunctional glands and contains the digestive fluid (Figure 1A) (Mithöfer, 2011). The bifunctional glands are responsible on the one hand for secretion of the digestive pitcher fluid and on the other hand for nutrient uptake generated by prey digestion (Figure 1B). All *Nepenthes* species possess a lid at the peristome, which is sometimes also involved in prey attraction and, in addition, protects the digestive fluid from rain that otherwise might dilute the digestive fluid (Clarke *et al.*, 2009, Grafe *et al.*, 2011).

1.2 Protein composition of *Nepenthes* pitcher fluid

Since the time of Darwin the proteolytic activity in the fluid of carnivorous pitcher plants has been assumed (Darwin, 1875). In recent years, researchers have focused on the elucidation of protein composition in the pitcher fluid by applying proteomic approaches; many proteins were identified (Hatano and Hamada, 2008, Mithöfer, 2011, Rottloff *et al.*, 2011, Hatano and Hamada, 2012, Buch *et al.*, 2014, Rottloff *et al.*, 2016). One of the most prominent proteins in the pitcher fluid is the pepsin-related aspartic protease nepenthesin. Nepenthesin I and nepenthesin II have been purified, cloned and biochemically characterized from different *Nepenthes* species (An *et al.*, 2002, Athauda *et al.*, 2004, Takahashi, 2007). Because of its special biochemical properties, nepenthesin is already applied in technological fields (Kadek *et al.*, 2014) and also used for therapeutic treatment (Rey *et al.*, 2016). Besides this, many other proteins were identified in the pitcher fluid, some might contribute to prey digestion, whereas others are not directly involved in the digestion process, but indirectly assist prey derived nutrient acquisition. Several classes of chitinases were also identified as components of the *Nepenthes* pitcher fluid (Eilenberg *et al.*, 2006, Hatano and Hamada, 2008, Rottloff *et al.*, 2011, Rottloff *et al.*, 2016). Further proteins in the pitcher

fluids from different *Nepenthes* species are identified like peroxidase, ribonuclease, S-like ribonuclease, glucanase and thaumatin-like proteins (Stephenson and Hogan, 2006, Hatano and Hamada, 2008, Hatano and Hamada, 2012, Nishimura *et al.*, 2014, Rottloff *et al.*, 2016). A combination of mass-spectrometry-based proteomics with transcriptome analysis of the pitcher performed in several *Nepenthes* species, allowed the identification of additional protein members. This includes, in addition to novel members of already reported protein classes, new proteins such as carboxypeptidases, α- and β-galactosidases, lipid transfer proteins and esterases/lipases (Lee *et al.*, 2016, Rottloff *et al.*, 2016).

Table 1: Pathogenesis-related (PR) proteins identified in the pitcher fluid of *Nepenthes* species and their PR family classification.

PR family	**Type**	**Species**	**Reference**
PR-1	PR-1 protein	*N. mirabilis*	Buch *et al*, 2014
PR-2	Glucanase	*N. alata*	Hatano & Hamada, 2008
PR-3	Chitinase I Chitinase IV	*N. khasiana* *N. alata*	Eilenberg *et al*., 2006 Hatano & Hamada, 2008
PR-5	Thaumatin-like protein	*N. singalana* *N. alata*	Rottloff *et al*., 2009 Hatano & Hamada, 2012
PR-7	Nepenthesin I and II Cysteine-protease	*N. gracilis* *N. ventricosa*	Athauda *et a*l., 2004 Stephenson & Hogan, 2006
PR-8	Chitinase III, endochitinase Chitinase III	*N. rafflesiana* *N. alata*	Rottloff *et al*., 2011 Hatano & Hamada, 2012
PR-9	Peroxidase III	*N. alata*	Hatano & Hamada, 2012
PR-10	Ribonuclease S-like-ribonuclease	*N. ventricosa* *N. bicalcarata*	Stephenson & Hogan, 2006 Nishimura *et al, 2014* Lee *et al*, 2016

All of the described proteins in pitcher fluids from different *Nepenthes* species can be classified into one of the known families of pathogeneses-related (PR) proteins (Table 1) (Mithöfer, 2011). Recently, even pathogenesis-related protein 1 (PR1) was identified from *Nepenthes alata* pitcher fluid, which in

this particular case showed antibacterial rather than antifungal activity (Buch *et al.*, 2014).

1.3 Plant pathogenesis related proteins

Pathogenesis-related proteins are defined as proteins which are inducible under pathological and related situations in at least one or more pathogen combinations. Numerous pathogenesis-related proteins have been detected and reported in many plant species (van Loon and van Strien, 1999). PR proteins are low molecular weight proteins, normally with size of 10 to 40 kDa. Biochemical properties such as stability in extreme pH and resistance to proteolytic cleavage allow them to survive under harsh conditions (Stintzi *et al.*, 1993). Based on their shared sequence homology, PR proteins are classified into different families. Classification into different groups of PR proteins is also based on migration in native polyacrylamide gel electrophoresis (PAGE), cross-reactivity with antiserum and with mRNA probes. Biological activity of the induced proteins also plays a role for classification into groups and isoelectric points of the proteins are considered for sub-classification in the group. 17 PR protein-families were reported (van Loon *et al.*, 2006).

Expression of PR proteins prevails at the basal level without pathogen attack (van Loon *et al.*, 2006). Different types of pathogens like bacteria, fungi and viruses induce PR proteins in plants (Sinha *et al.*, 2014). It is also observed that PR proteins are inducible under parasitic attacks by insects, nematodes and herbivores (van Loon *et al.*, 2006). Correlation of PR gene induction upon various attacks by a number of plant hormones is also reported (Kitajima and Sato, 1999, van Loon *et al.*, 2006). Furthermore, certain promoter regions, DNA sequences located upstream of gene coding regions, are closely related to the regulation of PR gene expression as a response to particular stimuli (Jung *et al.*, 2005, Spoel and Dong, 2012, Jiang *et al.*, 2015). These promoter regions of PR genes contain *cis*-acting regulatory elements,

specific binding sites for transcription factors, which are proteins involved in initiation and regulation of transcription.

1.4 Regulation of carnivory-related processes in carnivorous plants

To cope with stresses caused by different triggers including pathogen attack, insect herbivory and harsh environmental factors, plants possess inducible defense mechanisms (Mithöfer and Boland, 2012, Verma *et al.*, 2016). Here, plant hormones play a vital role in the regulation of complex signaling networks and downstream events in plants (Mithöfer and Boland, 2012). Very powerful endogenous signals required to mediate plant growth, development, defense related reactions as well as environmental adaptations are phytohormones, endogenous signaling compounds like jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), auxin (IAA), cytokinins (CKs) brassinolides or ethylene (Bari and Jones, 2009). JA and SA are the two main players in this complex network, especially in response to pathogen attack (Bari and Jones, 2009). JA normally regulates defense against herbivores and necrotrophic pathogens, whereas defense mechanisms, which target biotrophic and hemi-biotrophic pathogens, are induced by SA. SA is also involved in mediating the development of systemic acquired resistance (SAR) in response to pathogen-attack (Bari and Jones, 2009). Crosstalk between different plant hormones enables them to adapt the already existing defense mechanisms to particular conditions (Verma *et al.*, 2016).

Recently, the involvement of particular plant hormones in carnivory was demonstrated for some carnivorous plants. For example, JA-mediated prey capturing and digestion by forming an “outer stomach”, formed by tentacle and leaf-bending in *Drosera* was shown (Nakamura *et al.*, 2013, Krausko *et al.*, in press) Application of external jasmonates was sufficient to induce leaf-bending movement (Nakamura *et al.*, 2013). Later on, the importance of both mechanical and chemical stimuli to trigger JA and JA-isoleucine conjugate (JA-Ile) biosynthesis was indicated (Mithöfer *et al.*, 2014). Different stimuli

from the prey induced electrical signals and phytohormone response. Mechanical and chemical stimuli induced, first a local, and later, by repeated stimulation, a non-localized tentacle movement and enzyme secretion in sundew plants. Jasmonates play a central role in regulation of the digestive enzyme activity (Krausko *et al.*, in press).

In the fast trap-moving Venus flytrap, *Dionaea muscipula*, jasmonates control excitability, slow closure and external stomach formation by trap closing (Escalante-Perez *et al.*, 2011). JA signaling pathway is activated by electrical signal known as action potential (AP), created by prey contact with trigger hairs of the trap and a number of APs are needed to achieve differential expression of genes encoding prey-degrading hydrolases (Böhm *et al.*, 2016). External JA application to Venus flytrap increased the abundance of a cysteine endopeptidase, dionain, and the whole proteolytic activity in the digestive fluid, which was positively correlated with chemical stimulation from captured prey (Libiakova *et al.*, 2014). Very recently, the impact of JA on endocrine processes and chemo-sensing of nutrients was further demonstrated in *Dionaea* (Bemm *et al.*, 2016).

Jasmonates are derived from α-linolenic acid, which is a fatty acid originated from the chloroplast membrane. Linolenic acid is converted into 12-oxo-phytodienoic acid (OPDA) in the chloroplast and translocated to peroxisome. OPDA is reduced and β-oxidized to jasmonic acid (JA), which is exported to the cytosol. In the cytosol, JA is conjugated to the amino acid isoleucine to form JA-Ile, the most active form of JA. The receptor of JA-Ile, COI1 activates the downstream JA signaling by employing the ubiquitin/26 proteasome pathway that degrades jasmonate ZIM-domain (JAZ) proteins, repressor proteins that prevent the transcription of JA responsive genes (Wasternack and Hause, 2013). These JA responsive genes are associated with many defense-related biological processes in plants (Wasternack, 2007, Wasternack and Hause, 2013).

In case of *Nepenthes*, little is known about prey-derived signals, and the initiation, regulation, and induction of hydrolytic enzymes. Although, the

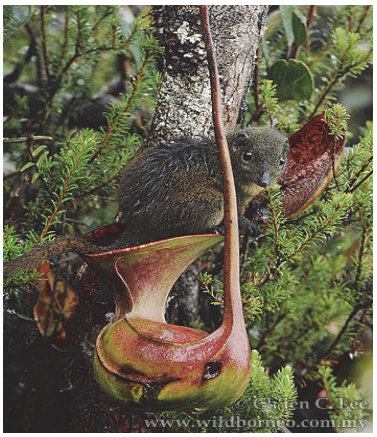
categorization of the pitcher fluid proteins in the PR protein families, strongly suggest hormonal participation in prey-derived nutrient supplementation, however an involvement of plant hormones also in the carnivory process in *Nepenthes* was unknown. Promoter structures of *Nepenthes* PR genes, which might be closely related to gene expression regulation were also not investigated till now.

1.5 Coprophagous features in *Nepenthes*

Most of the carnivorous plants including *Nepenthes* are specialized in insect prey capture and digestion. In addition, some distinct species of the genus *Nepenthes,* however, have developed an alternative cooperative lifestyle with animals. These are *Nepenthes hemsleyana*, (former name: *Nepenthes rafflesiana elongata), Nepenthes lowii* and *Nepenthes rajah* (Clarke *et al.*, 2010, Grafe *et al.*, 2011, Greenwood *et al.*, 2011, Schöner *et al.*, 2015). In these species a switch from prey digestion-derived sources to animal excrements has been documented (Clarke *et al.*, 2009, Grafe *et al.*, 2011, Greenwood *et al.*, 2011). Due to this switch, those *Nepenthes* species developed a mutualism-based coprophagous lifestyle. Mutualistic relationship has been, for instance, developed between *N. hemsleyana* and a bat, *Kerivoula hardwickii* (Figure 2), where bats are found roosting inside the pitcher. Recently, it was reported that reflective structures of *N. hemsleyana* acoustically attract bats for mutualism (Schöner *et al.*, 2015). A special morphological shape of the *N. hemsleyana* pitcher prevents the bats from falling into the pitcher fluid. Bats are defecating into the pitcher during their roosting activity (Grafe *et al.*, 2011). This, likely, results in plants getting access to high amount of nitrogen in the form of urea from bat urine since more than 70% (molar percentage) of bat urine consists of urea (Hales, 2014).

A

B



C

Figure 2. Mammals visiting specific *Nepenthes* pitchers. (A) Bat, *Kerivoula hardwickii* visiting *Nepenthes hemsleyana.* (B) Tree shrew, *Tupaia montana,* visiting *Nepenthes lowii*. (C) *Rattus baluensis* visiting *Nepenthes rajah*.

A similar mutualistic relationship was reported for *Nepenthes lowii* and the tree shrew, *Tupaia montana,* where the tree shrew feces represents a significant nitrogen source for *N. lowii* plants (Clarke *et al.*, 2009). Pitchers of *N. lowii* contain nectar-like substances inside the lid, which is the main attraction for the tree shrew. This nectar-like exudate is only accessible for tree shrews when they position their body on top of the pitcher, being ready for defecation at the same time (Clarke *et al.*, 2010). The same phenomenon was shown for *Nepenthes rajah* and several rat species. Thus, the pitcher shape is commensurate to the mutualistic partner that supports coprophagous behavior of these *Nepenthes* species. All described

interactions are good examples for multidirectional resource-based mutualism of carnivorous plants with mammals (Greenwood *et al.*, 2011). In all of these cases *Nepenthes* benefits from animal exudates. It was shown that *Nepenthes* plants get significant amount of nitrogen from this mutualism. Visiting activity of mutualistic partners significantly increased total foliar nitrogen content in *N. hemsleyana* and *N. lowii* plants. (Clarke *et al.*, 2009, Grafe *et al.*, 2011) However, the precise origin of nitrogen and biochemical background underlying this mechanism of nitrogen usage remains unclear.

1.6 Urea and urease in plant

Nitrogen (N) is one of the basic elements of proteins and nucleic acids (DNA, RNA). Lower N content per amino acid residue in plants compared to animals is observed, suggesting the evidence for an influence of environmental resource availability on proteomes of multicellular organisms. This observation is reflected in the decreased abundance on N-rich amino acids in the plant proteome (Elser *et al.*, 2006). Thus, nitrogen availability and metabolism is a vital factor for plants. Typically, biochemically accessible forms of nitrogen, generated with the help of microorganisms, are taken up by plant roots (Ibanez *et al.*, 2016). In agriculture, for example, urea serves as an excellent source for nitrogen. In animals, urea is excreted from the body as a metabolic waste product in order to remove toxic ammonia (Wright, 1995). The endogenous metabolic sources of urea are arginine and purine degradation. Nitrogen from internally and externally provided urea is only available for plants after urea hydrolysis (Wang *et al.*, 2008, Witte, 2011).

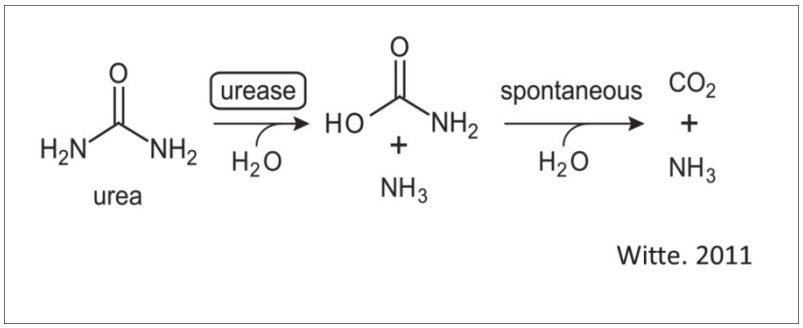


Figure 3. Urease reaction. Urea is enzymatically hydrolyzed to ammonia and carbamate. Further, ammonia and carbonic acid is generated subsequently by non-enzymatic decay of carbamate (adapted from Witte. 2011).

Therefore, the hydrolysis of urea to ammonia and carbon dioxide is necessary for making nitrogen accessible for assimilation (Figure 3). Urease is the only known nickel-dependent metalloenzyme and the only enzyme responsible for urea hydrolysis (Witte, 2011). Urease is produced by bacteria, fungi, yeast, and plants. Urea hydrolysis provides these organisms with a source of nitrogen. Plant and fungal ureases are homo-oligomeric proteins of 90-kDa identical subunits. Bacterial ureases however, are multimers of two- or three-subunit complexes. Nevertheless, bacterial urease share high sequence homology with plant urease (Balasubramanian and Ponnuraj, 2010). For the coprophagous lifestyle described for some *Nepenthes* species a role for urease is essential; however this topic still needed to be addressed.

1.7 Aim of the thesis

High adaptive plasticity enables plants to respond to changing environments. Some plants developed the carnivorous life style as an adaptation to limited nutrient acquisition in natural habitats. Carnivorous pitcher plant of the genus *Nepenthes* mainly rely on insect prey trapping and digestion and, hence, supply the plant with prey-derived nutrients. Additional coprophagous specialization of few *Nepenthes* species that interact with mammals seems to obtain high amount of nitrogen from their defecating mutualistic partners. The aim of this work was to study and identify exogenous and endogenous signals that mediate and regulate prey digestion in *Nepenthes.* In addition, the basis for coprophagous lifestyle in few *Nepenthes* species should be investigated.

The overall goal was to challenge the hypothesis that plant carnivory developed mainly from plant defense mechanisms and uses inherently existing tools. Therefore, the following aspects were studied in detail:

- ✓ Identification of prey-derived signals for the induction of genes encoding proteins for the pitcher fluid of *Nepenthes.*
- ✓ Analysis of the role for phytohormones in plant carnivory in *Nepenthes.*
- ✓ Promoter analysis for *Nepenthes* PR genes.
- ✓ Proof of principle of feces-derived origin of nitrogen in specialized coprophagous *Nepenthes hemsleyana*.

2. Manuscript overview

2.1 Manuscript I

Nepenthesin protease activity indicates digestive fluid dynamics in carnivorous *Nepenthes* plants

Authors: Franziska Buch, Wendy E. Kaman, Floris J. Bikker, Ayufu Yilamujiang, Axel Mithöfer
Status: Published, PLoS ONE 10(3): e0118853, 2015.
doi:10.1371/journal.pone.0118853

The aim of the study was to investigate the generation and induction of the aspartic protease, nepenthesin, in the pitcher fluid of *Nepenthes*. By employing FRET (fluorescent resonance energy transfer)-based technique that uses a synthetic fluorescent substrate, an easy and rapid procedure was established, which allowed the detection of protease activities in the digestive fluids of various *Nepenthes* species. Biochemical studies for heterologously expressed Nepenthesin II proved that the proteolytic property of the digestive fluid relied on the presence of aspartic proteases. By applying FRET-based approach, the induction and dynamics of nepenthesin in the digestive pitcher fluid could be studied directly upon challenge with insect prey or plant material. It was observed that proteolytic activity was induced not only by prey and litter but also by the phytohormone jasmonic acid in contrast to salicylic acid. The results suggest that jasmonate-signaling pathways are involved in plant carnivory.

F.B., A.M. conceived and designed the experiments; F.B., A.Y. performed the experiments; F.B., A.Y. and A.M. analyzed the data; W.E.K., F.J.B. contributed reagents/ materials/ analysis tools; F.B., W.E.K., F.J.B., A.Y. and A.M. wrote the paper.

2.2 Manuscript II

Slow food: Insect prey and chitin induce phytohormone accumulation and gene expression in carnivorous *Nepenthes* plants

Authors: Ayufu Yilamujiang, Michael Reichelt, Axel Mithöfer

Status: Published, Annals of Botany, 118: 369–375, 2016.
doi:10.1093/aob/mcw110.

The aim of this study was to investigate prey-depending induction of genes encoding proteins that are present in the pitcher fluid of *Nepenthes alata*, and the role of phytohormones in this process. It is demonstrated that insect prey as well as chitin is able to induce jasmonate phytohormones, which in turn can induce genes for digestive enzymes such as a protease, nepenthesin, or a chitinase. The results suggest that upon insect prey capture, a sequence of signals is initiated: (I) insect-derived chitin, (II) jasmonate as endogenous phytohormone signal, (III) induction of gene expression, (IV) digestive proteins expression. These results support the idea that carnivory evolved from plant defense.

A.Y. and A.M. conceived and designed the research; A.Y. performed the experiments; M.R. performed hormone measurements; A.Y., A.M. wrote the paper.

2.3 Manuscript III

Coprophagous features in carnivorous *Nepenthes* plants: a task for ureases

Authors: Ayufu Yilamujiang, Anting Zhu, Rodrigo Ligabue-Braun, Stefan Bartram, Claus-Peter Witte, Rainer Hedrich, Mitsuyasu Hasabe, Caroline R. Schöner, Michael G. Schöner, Gerald Kerth, Célia R. Carlini, Axel Mithöfer

Status: Submitted 30.01.2017, Nature Ecology & Evolution

Aim of this study was to investigate the molecular background and the role for urease in a coprophagous lifestyle in *Nepenthes*. The presence and enzymatic activity of a urease in *Nepenthes* plant tissues was shown. It was demonstrated that ^{15}N-enriched urea provided to *Nepenthes* pitchers is metabolized and its nitrogen is distributed within the plant. By applying PCR approach, urease cDNA from *N. hemsleyana* urease was isolated and heterologously expressed. Urease activity was only detectable by co-expression with *Arabidopsis* urease accessory proteins, UreD, UreF, UreG. A comprehensive phylogenetic analysis for eukaryotic ureases, including *Nepenthes* and five other carnivorous plants' taxa, identified them as canonical ureases and reflects the plant phylogeny. Hence, this study reveals the high adaptive plasticity in plants by developing a further specialized lifestyle from carnivory to coprophagy, on demand for nitrogen.

A.Y., C.R.C. and A.M. conceived and designed the research; A.Y., A.Z., S.B., and C.R.S., M.G.S, and C.-P.W. performed the experiments; R.L.-B. carried out phylogenetic analysis; R.H. and M.H. provided sequence information; C.R.S., M.G.S, G.K. established, grew and provided *N. hemsleyana* plant material; A.Y., A.M., and R.H. wrote the manuscript. All authors contributed to the manuscript.

Subject: NATECOLEVOL-17011237 Receipt of New Paper by Nature Ecology & Evolution
From: ecoevo@nature.com
Date: 31.01.2017 11:21
To: ayilamujiang@ice.mpg.de

Dear Dr. Yilamujiang,

Please note that you are listed as a co-author on the manuscript "Coprophagous features in carnivorous Nepenthes plants: a task for ureases" (reference number: NATECOLEVOL-17011237), which was recently submitted to Nature Ecology & Evolution.

The corresponding author is solely responsible for communicating with the journal and managing communication between co-authors. Please contact the corresponding author directly with any queries you may have related to this manuscript.

You can now use a single sign-on for all your accounts, view the status of all your manuscript submissions and reviews, access usage statistics for your published articles and download a record of your refereeing activity for the Nature journals. Please check your account regularly and ensure that we have your current contact information.

In addition, NPG encourages all authors and reviewers to associate an Open Researcher and Contributor Identifier (ORCID) to their account. ORCID is a community-based initiative that provides an open, non-proprietary and transparent registry of unique identifiers to help disambiguate research contributions.

Access your account

Many thanks,
NPG Applications Helpdesk
Nature Publishing Group

This email has been sent through the Springer Nature Tracking System NY-610A-NPG&MTS

3. Manuscripts

3.1 Manuscript I

PLOS | ONE

RESEARCH ARTICLE

Nepenthesin Protease Activity Indicates Digestive Fluid Dynamics in Carnivorous *Nepenthes* Plants

Franziska Buch[1], Wendy E. Kaman[2,3], Floris J. Bikker[3], Ayufu Yilamujiang[1], Axel Mithöfer[1]*

1 Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Hans Knöll Straße 8, D-07745, Jena, Germany, **2** Department of Medical Microbiology and Infectious Diseases, Erasmus MC, `s-Gravendijkwal 230, 3015 CE, Rotterdam, The Netherlands, **3** Department of Oral Biochemistry, Academic Centre for Dentistry Amsterdam, University of Amsterdam and VU University Amsterdam, Gustav Mahlerlaan 3004, 1081 LA, Amsterdam, The Netherlands

* amithoefer@ice.mpg.de

OPEN ACCESS

Citation: Buch F, Kaman WE, Bikker FJ, Yilamujiang A, Mithöfer A (2015) Nepenthesin Protease Activity Indicates Digestive Fluid Dynamics in Carnivorous *Nepenthes* Plants. PLoS ONE 10(3): e0118853. doi:10.1371/journal.pone.0118853

Academic Editor: Dawn Sywassink Luthe, Pennsylvania State University, UNITED STATES

Received: October 29, 2014

Accepted: January 16, 2015

Published: March 9, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The authors received no specific funding for this work.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Carnivorous plants use different morphological features to attract, trap and digest prey, mainly insects. Plants from the genus *Nepenthes* possess specialized leaves called pitchers that function as pitfall-traps. These pitchers are filled with a digestive fluid that is generated by the plants themselves. In order to digest caught prey in their pitchers, *Nepenthes* plants produce various hydrolytic enzymes including aspartic proteases, nepenthesins (Nep). Knowledge about the generation and induction of these proteases is limited. Here, by employing a FRET (fluorescent resonance energy transfer)-based technique that uses a synthetic fluorescent substrate an easy and rapid detection of protease activities in the digestive fluids of various *Nepenthes* species was feasible. Biochemical studies and the heterologously expressed Nep II from *Nepenthes mirabilis* proved that the proteolytic activity relied on aspartic proteases, however an acid-mediated auto-activation mechanism was necessary. Employing the FRET-based approach, the induction and dynamics of nepenthesin in the digestive pitcher fluid of various *Nepenthes* plants could be studied directly with insect (*Drosophila melanogaster*) prey or plant material. Moreover, we observed that proteolytic activity was induced by the phytohormone jasmonic acid but not by salicylic acid suggesting that jasmonate-dependent signaling pathways are involved in plant carnivory.

Introduction

Charles Darwin's work "Insectivorous Plants" published in 1875 [1] still contains much of what we know about those specialized plants. However, Darwin never worked with the genus *Nepenthes*, which is distributed primarily in Southeast Asia. *Nepenthes* pitcher plants have so-called pitfall-traps that are divided into *i)* an upper part representing the attraction zone, *ii)* a part in the middle representing the slippery zone, and *iii)* a lower part, the digestion zone.

Pitchers are filled with a digestive fluid, or enzyme cocktail, to digest caught prey [2,3]. Even closed pitchers have such a fluid, which is both plant-derived and sterile [4]. Since Darwin, scientists have known that hydrolytic activity—in particular, proteolytic activity—is present in insectivorous plants. In addition to proteases, the digestive fluid of *Nepenthes* spp. is known to contain various esterases, phosphatases, ribonucleases and different chitinases (e.g. [2,3,5,6,7,8]). Proteases in digestive fluid from several species of *Nepenthes* have also been described early [9], purified and characterized (e.g. [10,11,12]). However, only An et al.[13] cloned nepenthesins from the pitcher tissue of *N. alata*. Two years later nepenthesin I and II from *N. distillatoria* were purified and characterized [14]. After the nepenthesin cDNAs were cloned from *N. gracilis* pitchers [14], these proteases were identified as members of a new subfamily of aspartic proteases [14,15,16]. In addition, Stephenson and Hogan [17] reported a cysteine protease in *N. ventricosa*.

Otherwise, little is known about the regulation and induction of hydrolytic enzymes involved in the digestive process in carnivorous plants. In recent years, gene induction analyses were carried out on the tissue of the pitcher to search for hydrolytic enzymes [5,6,18]. This also holds true for the very prominent aspartic proteases in *Nepenthes* [13], although the proteolytic activity in the pitcher fluid represents an ideal target to follow and study dynamic processes during carnivory in *Nepenthes* pitfalls. But up to now, the low amounts of enzymes in the pitchers have made it impossible to analyze changes in the digestive fluid depending on developmental stages of the pitcher or in response to prey capture.

Here, we report on the introduction of a new technique, the highly sensitive FRET (fluorescent resonance energy transfer), for the direct, easy and rapid detection and characterization of protease activity in the digestive fluids of *Nepenthes*. Using a synthetic fluorogenic substrate, i.e. FRET peptide, we investigated the dynamics of protease activity in response to various stimuli. In addition, we cloned and expressed the proteases involved in the enzymatic reaction, nepenthesin I and II.

Material and Methods

Organisms and culture conditions

Nepenthes plants *(N. mirabilis*, *N. alata)* were grown in the greenhouse of the Max Planck Institute for Chemical Ecology in Jena under controlled conditions. The plants were cultivated in a growth chamber with a photoperiod of 15 h light/9 h dark, day/night temperature of 18–20° C/16–18°C and humidity about 55%. Every day, plants were sprayed and every second day they were watered with rain water.

Both tissue from the lower part of the pitchers and pitcher fluid from *N. mirabilis* and *N. alata* were used for this study. As well, the pitcher fluid of other *Nepenthes* species (*N. reinwardtiana*, *N. distillatoria*, *N. wittei*, *N. hookariana*, *N. boschiana*, *N. maxima*, *N. eymae* and the hybrid *N. alata x N. ventricosa*), which were grown in the greenhouses of the Botanical Gardens in Jena and Munich, were used for fluorescence intensity measurements. The pitcher fluid samples were collected from closed pitchers using a sterile syringe, from open pitchers by pouring fluid directly into 15 ml sterile Falcon tubes.

Spodoptera frugiperda Sf9 cells, derived from the pupal ovarian tissue of the insect and originating from the IPLBSF-21 cell line (Invitrogen, Darmstadt, Germany), were used for the transfection and expression of the *Nepenthes* aspartic proteinases, nepenthesin (Nep) I and II. They were cultured at 27°C in Sf-900 II serum-free medium (sf- medium) (Gibco) in presence of 50 μg/ml gentamycin.

Measuring protease activity with fluorescent substrate and FRET

Using a small and highly specific FRET peptide substrate (FITC(Ahx)-Val-Val-LysDbc), encoded as PFU-093 by Kaman et al. [19,20], we measured the proteolytic activity of the pitcher fluid. PFU-093, one of many substrates developed to study the presence of bacteria *in situ* (saliva, sputa, serum), was designed with fluorescein isothiocyanate (FITC) operating as a fluorophore and LysDbc acting as its quencher. When both molecules are physically close, the connection made by the two-amino acid bridge quenches the fluorescence and no activity can be detected (Fig. Aa in S1 File). However, when proteolytic activity separates the fluorophore and quencher, fluorescence intensity can be measured using a microplate reader (Tecan infinite M200, Männedorf, Switzerland) (Fig. Ab in S1 File). We mixed 50 μl of *Nepenthes* pitcher fluid, 49 μl pure water (Gibco) for dilution and 1 μl of 80 μM PFU-093 in black 96-well microtiter plates (Greiner Bio-one GmbH, Frickenhausen, Germany), and measurements were taken for up to 11 h. The fluorescence activity was measured at 42°C and an excitation/ emission wavelength of 485 nm/530 nm.

Biochemical studies

The impact of pH on the FRET method was tested by incubating 500 μl *N. alata* pitcher fluid mixed with 245 μl pure water and 5 μl of 80 μM PFU-093. After 10 h at 42°C, 50 μl each of this mixture was transferred into 10 different wells and mixed with another 50 μl of various 30 mM buffer solutions (pH 2, pH 3, pH 4, up to pH 10) with H_2O as a control. Then fluorescence was measured and the following categories were used: for an acidic range, citrate buffer from pH 2 to 4.9; for an acid-base balance, phosphate buffer from pH 4.9 to pH 8.5; and for a basic pH range, glycine buffer from pH 8.5–10.

To verify the stability of the reaction, digestive fluid was pre-incubated with 30 mM citrate buffer pH 4.0 for 10 h at 42°C; as a control, pre-incubation was done with water. For fluorescence measuring, 50 μl of this mixture was either mixed with 30 mM phosphate buffer pH 8 or with 30 mM citrate buffer pH 4.

Inhibitor tests were performed by mixing 50 μl of pitcher fluid, 1 μl of 80 μM PFU-093 and various inhibitors at different concentrations: including 100 μM phenanthroline, 100 μM AEBSF, 20 μM E-64 and 100 μM pepstatin A. Samples were mixed with pure water to 100 μl final volume and measured as described earlier, including the inhibitors solvents (DMSO, MeOH, H2O) and pure pitcher fluid for control.

Determination of substrate cleavage site

To determine the cleavage site of PFU-093 substrate cleaved by *Nepenthes* aspartic proteases, Nep I/II, HPLC-MS was performed. For this measurement, 495 μl pitcher fluid of both *N. mirabilis* and *N. alata*, as well as 5 μl of 80 μM PFU-093 substrate, was added to 1.5 ml safe-lock tubes (Eppendorf AG, Hamburg, Germany) and incubated for 11 h at 42°C. This mixture was concentrated by drying and subsequently resolved in 60 μl H_2O. Of the concentrated probe, 20 μl was injected into a Dionex UltiMate 3000 HPLC system, equipped with a Kinetex C18 column and connected to a Thermo Fisher LTQ for MS. ESI-MS in positive ion mode was used for searching three different fragment masses; *m/z* 397.47, *m/z* 496.28, *m/z* 619.8 (Fig. Ac in S1 File).

RNA preparation and cDNA synthesis

Tissue samples were collected by cutting a pitcher for homogenization, using only the lower third, i.e. the part that possesses the multicellular glands [21]. Total RNA from the lower part

of one *N. mirabilis* pitcher was isolated using the InviTrap Spin Plant RNA Mini Kit (Stratec Molecular, Berlin, Germany) following the manufacturer's protocol. For RNA cleanup and concentration RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) was used. First-strand cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) as well as up to 5 μg total RNA according to the specified protocol.

Rapid amplification of cDNA ends (RACE), cloning and sequencing

Previously sequenced *N. gracilis* and *N. alata* nepenthesin mRNA transcripts (GenBank accessions: *Ng*NepI, AB114914; *Ng*NepII, AB114915; *Na*NepI AB266803) were aligned using the CLUSTAL-W multiple sequence alignment program (available from: http://www.genome.jp/tools/clustalw/) and assessed for percent sequence identity. Specific primers for both nepenthesin I and II were designed in regions where 100% sequence identity was found (Table A in S1 File, primer sequences 1–4). These primers were used to amplify fragments of cDNA sequences using *N. mirabilis* cDNA as a template for the PCR reactions. Subsequently, the amplified products were cloned into a pCR 2.1-TOPO vector following the described TOPO TA cloning protocol (Invitrogen, Darmstadt, Germany) and sent for sequencing (Eurofins MWG Operon, Ebersberg, Germany). The resulting partial sequences were used to design *N. mirabilis* Nep I and II RACE primers via version 4.0.0. of Primer3Web software [22] (Table A in S1 File, RACE primer sequences 5–8). For generating 5'- and 3'- RACE-Ready cDNA, the manual of the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, Canada/ USA) was followed. The generation of 5' and 3' cDNA fragments of Nep I and II was described as 5'-RACE and 3'- RACE PCR reactions in the manufacturer's manual. To clean and concentrate DNA, DNA Clean & Concentrator-5 (Zymo Research, Irvine, CA, USA) was used. The resulting amplified products were cloned into a pCR 2.1-TOPO vector following the described procedure (see above). The resulting plasmids were sequenced by Eurofins MWG Operon. The complete Nep I and II cDNA sequences were identified by using the DNASTAR Lasergene Software SeqMan Pro (Madison, WI, USA). Subsequently, known Nep I and II protein sequences from *N. gracilis (Ng)* and *N. alata (Na)* were compared with the sequences of *N. mirabilis* (*Nm*). Protein sequences from NCBI Genbank were aligned by using "Jalview- *Open Source* Bioinformatics"- Software [23] and "MegAlign (DNASTAR)"- software version 10.1.2.

Expression in insect cells and Western blot

For functional identification, cDNA was amplified with primer sequences 9–10 for Nep I and primer sequences 11–12 for Nep. II (Table A in S1 File) to obtain an open reading frame (ORF) that lacks the predicted signal peptide (SP). The cDNA from Nep I and II was subcloned in pMIB/V5-His vector A (Invitrogen, Darmstadt, Germany) for transfection into Sf9 cells using lipid-mediated transfection. Cells were transfected in 60-mm diameter Petri dishes with 4 μg of plasmid DNA using Insect Gene Juice (Novagen, Nottingham, UK) as a transfection reagent. After 48 h, cells were split 1:5 in a 60-mm diameter Petri dish and selected with 80 μg/ ml blasticidin until they reached confluency. Expression was analyzed by Western blot using the anti-V5 horseradish peroxidase antibody (Invitrogen).

Auto-activation of *Nepenthes mirabilis* nepenthesin II

Recombinant nepenthesin II (*Nm*NepII/Sf9) from *N. mirabilis*, expressed in Sf9 culture medium, was dialyzed against pure H_2O (Slide-A-Lyzer Dialysis Cassettes; 10.000 MWCO, Thermo Fisher Scientific, Bonn, Germany) for 24 h; the same was done with Sf9 culture medium (Sf9cm) only for a control. Next, 500 μl of the dialyzed *Nm*NepII/Sf9 and 500 μl of Sf9cm were incubated at room temperature; 60 μl 1 M glycine- HCl- buffer (pH 4) was added to promote

auto-cleavage of the protease and to eliminate the pro-peptide (in Nep II protein sequence at position 73) by acidification. After 24 h of incubation at pH 4, 100 µl of 100 mM Tris- HCl buffer pH 8.5 was added to both mixtures to achieve a more basic pH range to measure fluorescence. Furthermore, 500 µl of *Nm*NepII/SF9 and SF9cm was measured without pre-incubation in pH 4 buffer. Fluorescence intensity measurements of each mixture were taken every 5 min for 6 h in a microplate reader in a black 96-well plate with 5 technical replicates.

Protease activities in *Nepenthes* pitcher fluids induced by different treatments

To induce protease activity, the pitcher fluids of two *Nepenthes* species, *N. alata* and *N. mirabilis*, were supplemented with *i)* fruit flies, *Drosophila melanogaster*, as insects represent the natural food, and *ii)* a piece of *Nepenthes* leaf as plant-derived food material. In addition, the phytohormones *iii)* jasmonic acid (JA), and *iiii)* salicylic acid (SA) were also tested for their ability to induce protease activity. In both cases the phytohormones were injected directly into the pitcher fluid.

Fluorescence activity was measured first for control values before feeding/ treating *Nepenthes* plants (time point 0 h) and subsequently at different time points (from 24 h to 240 h) samples were taken and analyzed for proteolytic activity using the PFU-093 substrate. Ten *D. melanogaster* flies, two pieces of 1.5 x 1.5 cm damaged *Nepenthes* leaf, and approximately 200 µM each of JA and of SA were added to the pitcher fluid. Usually, three replicates were done; duplicates for feeding with *Nepenthes* leaf material, and four repeats in the case of SA. Experiments were performed under semi-sterile conditions by covering the pitchers with gauze before plants opened their lids (Fig. 1).

As an additional experiment, the pH regulation in the digestive fluid was observed *in vivo*: 5 pitchers were each challenged with 40 *D. melanogaster* flies. The pH of digestive fluids was measured before (0 h) and at different time points (between 2 and 192 h) after treatment by immersing test strips directly into the pitcher fluid. Measurements included two control pitchers without fruit flies. In addition, 200 µl samples taken at various time points (0, 96, 144, 192 h) were tested for protease activity by incubating 50 µl pitcher fluid mixed with 39 µl H_2O and 1 µl of 80 µM PFU-093 substrate per reaction for 5 h at room temperature. Afterwards, probes were mixed with 10 µl of 100 mM Tris- HCl buffer, pH 8.5, to a final volume of 100 µl and pH 8 in each sample, and subsequently, samples were measured in a microplate reader. The removed volume was replaced with 25 mM KCl.

Results and Discussion

Proteolytic activity in *Nepenthes* pitchers

Employing the FRET technique with PFU-093 as substrate, proteolytic activity was detected in the pitcher fluid of all ten *Nepenthes* species tested although at different intensities (Fig. 2A). Because fluids from closed pitchers were found to be sterile and contain only plant-derived components [4,24,25], the digestive fluids from closed and open pitchers of *N. alata* were also compared. In Fig. 2B, proteolytic activity was detectable in both samples, indicating that the activity in the closed pitcher originated exclusively from the plant itself. Based on that, sterile digestive fluids from closed or newly opened pitchers (kept semi-sterile with gauze, Fig. 1B) were used for further measurements.

The pH of the substrate cleavage reaction was close to 8. This contradicted published results for pitcher fluids containing proteases, describe acidic pH ranges [13,25]. Therefore, the proteolytic reaction was performed at neutral pH (water) for 10 h, and afterwards the pH was

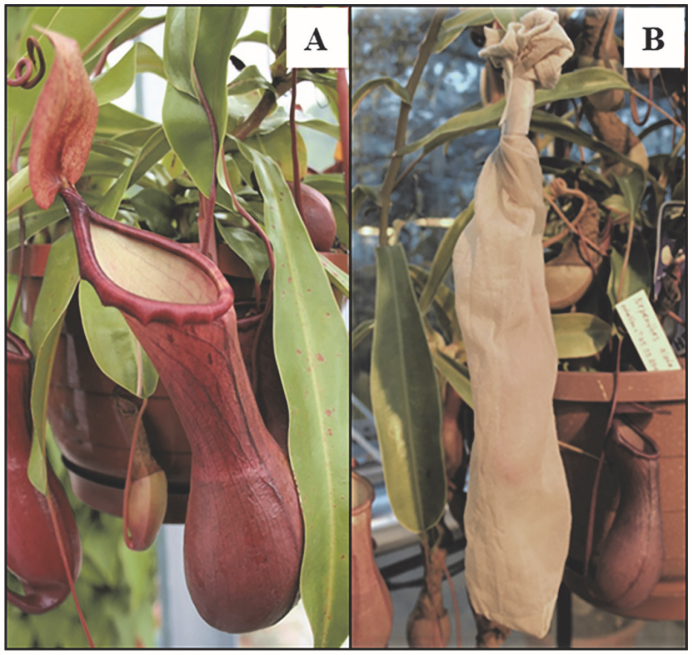


Fig 1. *Nepenthes alata* pitchers. A Without and **B** covered with gauze. Pitchers/ pitcher fluid of *Nepenthes* species can be kept under semi-sterile conditions by using gauze.

doi:10.1371/journal.pone.0118853.g001

adjusted with buffers of high molarity before the final fluorescence was measured. In the control, only water was added. The result (Fig. Ba in S1 File) suggests that the PFU-093 intrinsic fluorescence was quenched under acidic conditions and depended on decreasing pH levels, whereas at neutral and basic pH levels it was detectable. This forced us to keep or adjust the pH value between 7 and 8 in all fluorescence measurements.

In order to analyze whether the proteolytic reaction takes place not only at neutral pH but also under acidic conditions, a subsequent incubation experiment at pH 4 was undertaken. Results revealed that it was possible to restore measurable fluorescence simply by adding a strong buffer and adjusting the pH of the sample to 8 directly after incubation (Fig. Bb in S1 File). This result also demonstrates that the proteases, which cleave the artificial substrate, are stable and active at acidic as well as slightly basic pH ranges, suggesting that these enzymes act like the aspartic proteinases nepenthesin I and II, purified and characterized from several *Nepenthes* species [9,10,11,12,13,14,15,16,17,26].

Since the most prominent proteases in *Nepenthes* pitcher fluid are nepenthesins, it was conceivable that the activity we measured was due to those aspartic proteases. To challenge this idea, various inhibitors with specificities against different types of proteases were used in combination with the proteolysis assay. Inhibitors for metalloproteases (phenanthroline), serine-proteases (AEBSF), and cysteine-proteases (E-64) showed no or minor effect (Fig. 3). In contrast, the proteolytic activity in the pitcher fluid is strongly inhibited (4.0 to 4.5% remaining activity) by the aspartic protease inhibitor pepstatin A. This result is in agreement with results obtained for pepstatin-inhibition of nepenthesin activity in *N. alata* [13] and *N. distillatoria* [14].

A detailed analysis of the cleavage reaction determined the cleavage site in the substrate (Figs. Ab and Ac in S1 File) shows the putative cleavage sites and the resulting products). An

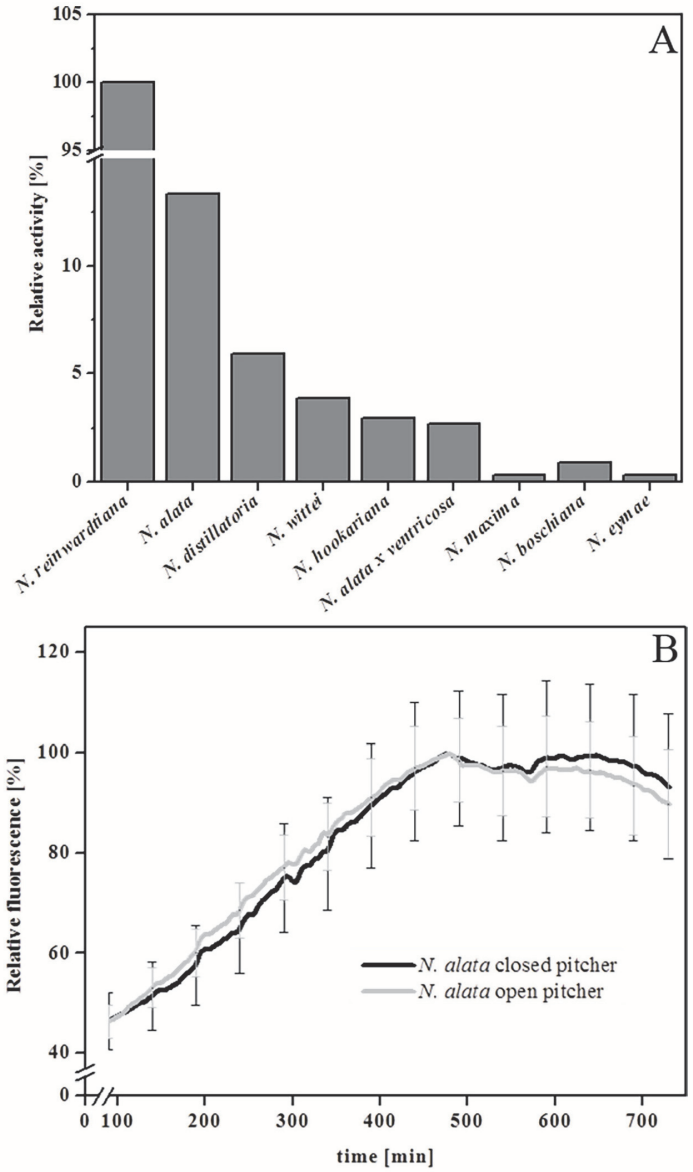


Fig 2. Proteolytic activity in *Nepenthes*. **A** Samples of the pitcher fluid of ten different *Nepenthes* species were investigated for their PFU-093 cleaving activity. **B** Kinetics of proteolytic PFU-093 cleaving activity in pitcher fluid from open (grey line) and closed (black line) pitchers of *Nepenthes alata*. Relative fluorescence was measured over 12 h at 42°C.

doi:10.1371/journal.pone.0118853.g002

HPLC-MS analysis of the PFU-093 cleavage products after treatment with *Nepenthes* proteases proved that, as expected, the substrate was cleaved between the two valines (Fig. 4). Although the molecular weight of the substrate was 1098 g/mol (Fig. 4B), a fragment ion with *m/z* 620, corresponding to the FITC(Ahx)-Val fragment (Fig. 4C), was detected after flies were digested

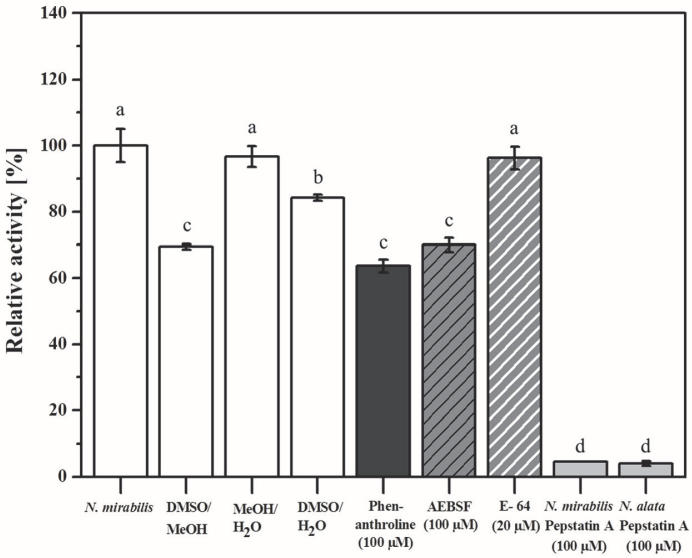


Fig 3. Inhibitor experiments. Different protease inhibitors—phenanthroline, AEBSF, E-64 and pepstatin A—were tested for their ability to inhibit protease activity responsible for PFU-093 cleavage. Individual working concentrations are indicated in brackets. Pitcher fluid from *N. mirabilis* without any inhibitor was used as a control (from left, first bar). Additional controls were carried out with the particular solvents of the inhibitors (DMSO, MeOH, H_2O). Statistics was done using one-way ANOVA, All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method), $P<0.05$; different letters indicate significant differences.

doi:10.1371/journal.pone.0118853.g003

in pitcher fluid from both *N. mirabilis* and *N. alata* (Fig. 4A). Searching for a fragment m/z = 397–398, we found a smaller peak in the chromatogram (Fig. 4B, green line) at R_t 17.1 min. This peak likely represents the "LysDbc" cleavage fragment and indicates that the second valine was also cleaved off. No fragment representing "Val-LysDbc" (m/z = 497–498) was detected.

Cloning and heterologous expression of nepenthesin

To confirm that nepenthesin is the active protease involved in PFU-093 degradation, the cDNAs of nepenthesin I and II were cloned. First RNA was isolated from *N. mirabilis* pitchers.

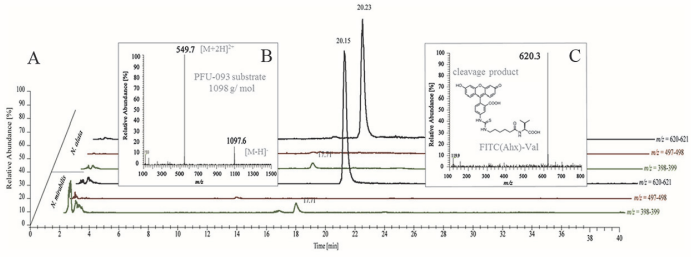


Fig 4. Determination of protease cleavage site. A sample of PFU-093 after digestion with pitcher fluid proteases (*N. mirabilis* and *N. alata*) was analyzed by HPLC-MS. **A** Single ion chromatograms for mass ranges m/z = 620–621 amu (black line), m/z = 497–498 amu (red line) and m/z = 398–399 amu (green line). **B** Mass spectrum of PFU-093 (FITC(Ahx)-Val-Val-LysDbc). **C** Mass spectrum of the FITC(Ahx)-Val fragment after cleavage of PFU-093, FITC(Ahx)-Val-Val-LysDbc (MS was done with ESI in the positive ion mode).

doi:10.1371/journal.pone.0118853.g004

After synthesizing cDNA, RACE PCR was further used to isolate the missing 5' and 3' regions. The resulting complete cDNA sequences of both Nep I and II, which include 5'- and 3'- untranslated regions, had 1570 and 1610 base pairs, respectively. The ORFs for Nep I and II both had 1314 base pairs, encoding 437 amino acid sequences (GenBank accessions AFV26024 (Nep I), AFV26025 (Nep II). Both Nep I and II possess a signal peptide for secretion which is predicted to have 24 amino acids (prediction was made using SignalP 4.1 Server [27]. The predicted molecular weights of Nep I and II without signal peptides were calculated with 43.7 and 43.5 kDa, respectively. In addition, Nep I possesses seven predicted N-glycosylation sites used NetNGlyc 1.0 Server [28], whereas Nep II possesses only two. These observations support the fact that the glycosylation of nepenthesin proteins has been previously observed in *Nepenthes distillatoria* [14].

Percent identity between *N. mirabilis* (*Nm*NepI/II), *N. alata* (*Na*NepI/II) and *N. gracilis* (*Ng*NepI/II) amino acid sequences, was determined by alignment by using the CLUSTAL-W software program (see above) (Fig. C in S1 File). We found that the Nep I and Nep II sequences had rather high percent identity: 94.1% (*Nm*NepI:*Ng*NepI), 99.1% (*Nm*NepI:*Na*NepI) and 96.1% (*Nm*NepII:*Ng*NepII). However, the maximum percent identity is about 66% when sequences of *Nm*NepI are compared to those of *Nm*NepII. In Fig. C in S1 File the predicted signal peptide (SP) cleavage sites,predicted by SignalP- software [27] are shown to be located between amino acid positions 24 and 25: THS/TS. Thus, the active proteins start with the amino acids "NGPS" (Nep I) and "QSSS" (Nep II), respectively. Between the SPs and the active protein, propeptide sequences [14] were detected (Fig. C in S1 File). The *N. mirabilis* proteases were found to be typical nepenthesin-aspartic proteases: on one hand they lack the PSI (plant-specific insertion), which is typical for vacuolar APs [29], and on the other hand, they possess a special insertion assigned to residues 148–169 and known as 'the nepenthesin- type AP (NAP)-specific insertion' [14]. This insertion contains four additional cysteine residues (arranged pairwise to form disulphide bonds), shown in Fig. C in S1 File as yellow, dark green, light green and orange; these residues contribute to the primary structure [14,15] and precede the tyrosine residue that is shown as a small green box above the sequence. The other two cysteine residues are red and light red. The two catalytic aspartic acid residues are shown as small black boxes.

For heterologous expression in insect Sf9 cells, primers (see above) were used to amplify the ORFs of Nep I and II excluding their native signal peptide and stop codon. These fragments were cloned in frames with the sequence corresponding to the signal peptide from the bee mellitin at the 5'-end and with a V5-$(His)_6$ tag at the 3'-end. After 72 h, culture medium was harvested and tested by Western blotting, using an anti-V5 horseradish peroxidase antibody that showed the expression of *Nm*NepII/Sf9 (Fig. D in S1 File) but not of *Nm*NepI/Sf9. However, *Nm*NepII/Sf9 did not show proteolytic activity.

The proteolytic activity of *Nm*NepII/Sf9 was activated by an acid-mediated cleavage of the expressed propeptide as described for nepenthesin-1 from *N. gracilis* expressed in *E. coli* [30]. After being activated, *Nm*NepII/Sf9 was incubated for 24h at pH 4; then its protease activity could be measured (Fig. 5) at different protein concentrations (lines 1–3). The control without the presence of PFU-093 substrate (line 4) and the *Nm*NepII/Sf9 control lacking auto-activation (line 5) show no detectable activity. The activity of *Nm*NepII/Sf9 confirms our initial assumption that the aspartic proteases in the pitcher fluid, i.e. nepenthesins, are responsible for cleaving the fluorescent substrate.

To see a direct correlation between proteolytic activity and the pH of the pitcher fluid, an additional experiment was done in which pitchers were supplemented with fruit flies and pH was determined every time samples were taken (Fig. 6A). In Fig. 6A, the pH of pitcher fluid decreased after flies were added to the fluid, and a value of 4 was reached in only 48 h, a value of around 3 after 96 h. pH continued decreasing until 192 h. Just as nepenthesins become auto-

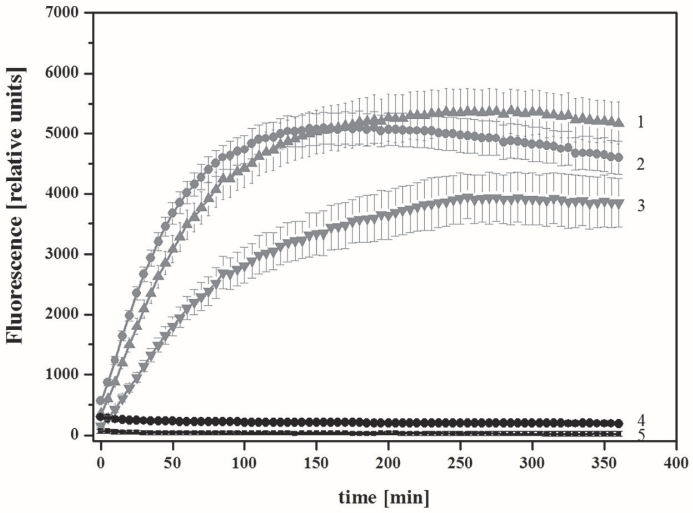


Fig 5. Proteolytic activity of recombinant *N. mirabilis* Nepenthesin II (*Nm*NepII/Sf9). PFU-093 fluorescence was measured over 6 h every 5 min after pre-incubation at pH 4 for 24 h for auto-activation. Different concentrations of *Nm*NepII/Sf9 were tested with constant concentrations of PFU-093 substrate. Line 1: 99 µl of *Nm*NepII/Sf9; line 2: 49 µl *Nm*NepII/Sf9; line 3: 24.5 µl *Nm*NepII/Sf9. All were mixed with pure H_20 and 1 µl of 80 µM fluorescent substrate to a total volume of 100 µl per well. Controls: line 4: 99 µl *Nm*NepII/Sf9 and H_2O without the addition of fluorescent substrate; line 5: 99 µl *Nm*NepII/Sf9 and 1 µl of 80 µM PFU-093 without pre-incubation/ auto-activation in pH 4 glycine-buffer.

doi:10.1371/journal.pone.0118853.g005

activated when the surrounding medium is acidified (Fig. 6) [18], so protease activity increases when pitcher fluid is acidified by the addition of *D. melanogaster*. In Fig. 6B, a significant increase of protease activity is shown 96 h after the addition of fruit flies, confirming the role of nepenthesins as active proteases.

Induction of nepenthesins

FRET is a tool to directly analyze proteolytic, namely nepenthesin, levels in the digestive fluids of *Nepenthes*. Compared with others (e.g. [13]), this method is faster, more convenient, and specific for aspartic proteases. Moreover, kinetics can be easily measured with the same sample. The direct analysis of nepenthesins as one of the most prominent enzymes in the digestive fluid of *Nepenthes* pitchers can be seen as the leading-activity for changes in the pitcher fluid activities that occur, for example, after the plants capture prey. As recently shown for the carnivorous plant *Dionaea muscipula* [31,32], besides nepenthesins the presence of more proteases can be expected. However, in *Nepenthes*, only in *N. ventricosa* a cysteine proteinase activity was described in the pitcher fluid and a cDNA encoding a putative cysteine protease was cloned from pitcher tissue [17].

Up to now, the induction of hydrolytic enzymes in the pitcher was investigated only indirectly by gene induction analyses of the pitcher tissue [5,6,13,33]. Now, we are able to follow such dynamic processes simply by determining the Nep protease activity. Such analyses require pitchers of *N. mirabilis* and *N. alata* to first be treated by supplementing them with prey such as *D. melanogaster*. This treatment resulted in a significant increase in proteolytic activity after 48 h (Fig. 7A). With some delay, the same effect was visible after supplementing the pitchers with *Nepenthes* leaf material (Fig. 7B). Although the latter finding might suggest a kind of 'cannibalism' in *Nepenthes*, this was not surprising because it is known that carnivorous plants

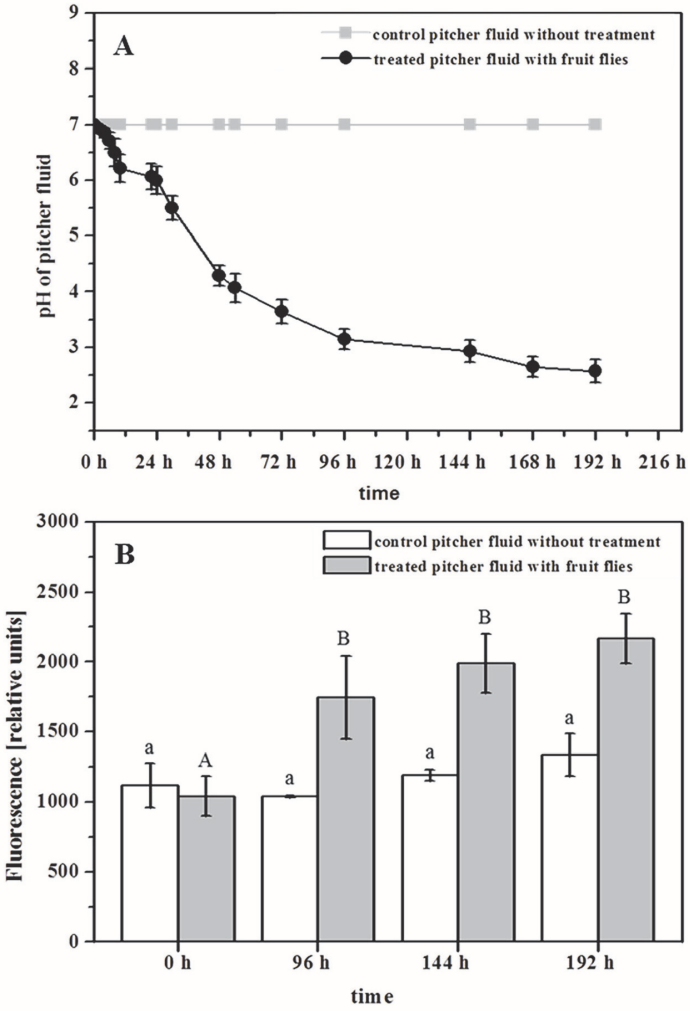


Fig 6. Influence of *Drosophila melanogaster* on pitcher fluid- pH and nepenthesin levels. Pitchers were supplemented with 40 fruit flies each. **A** The pitcher fluid pH was continuously determined at different time points until 192 h after treatment (black line); control without treatment (gray line). **B** Protease activity was measured before (0 h) and after the addition of fruit flies (96, 144, 192 h; gray bars) by using PFU-093 substrate for each sample. Measurement included 2 control pitchers (white bars). For **B** One-way ANOVA, $P<0.05$, Post Doc Test_*SNK*, was performed.

doi:10.1371/journal.pone.0118853.g006

actually take whatever they get for their nutrition. For example, *N. ampullaria* is specialized to capture leaf litter from the canopy above [34]. With any type of organism that falls in the trap all sources of nutrients become available for the plant and justify the induction of various hydrolyzing enzymes.

Recently, it has been shown for different carnivorous plant genera that defense-related phytohormones are involved in the trapping process, e.g. for *Drosera capensis* [35,36] as well as in the digestion process, e.g. in *D. muscipula* [37,38]. In order to follow up those studies, we investigated the effects of phytohormones in *Nepenthes* on protease activity. Interestingly, when

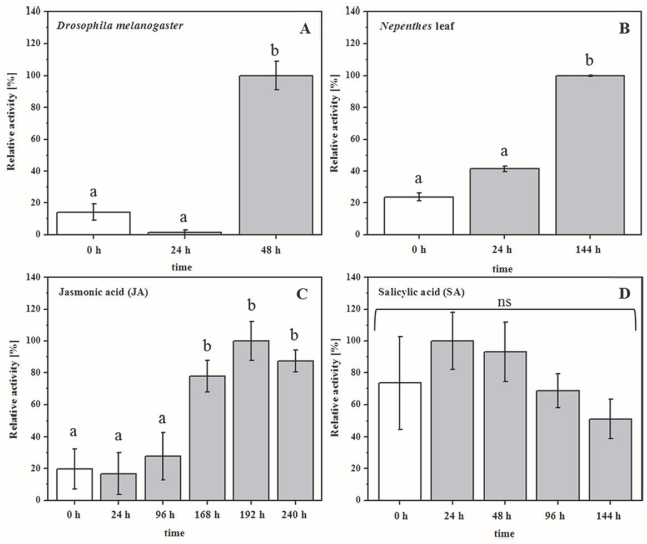


Fig 7. Induction of nepenthesin protease activity in *Nepenthes mirabilis* pitcher fluid. Proteolytic activity was measured with PFU-093 substrate at different time points after various treatments: **A** supplementation with *D. melanogaster* ($n = 3$); **B** supplementation with *Nepenthes* leaf ($n = 2$); **C** injection of jasmonic acid (200 μM end concentration) ($n = 3$) **D** injection of salicylic acid (end concentration 200μM) ($n = 4$). Statistics employed one-way ANOVA, $P<0.05$, Post Hoc Tests_*SNK* **(A, C, D),** Dunnett T3 **(B).**

doi:10.1371/journal.pone.0118853.g007

phytohormones were added to the pitcher, only the addition of jasmonic acid (JA), not salicylic acid (SA), significantly increased the Nep- activities (Fig. 7C and 7D). These results support the hypothesis that carnivory in plants might have evolved from defensive reactions against pathogens or herbivores [7,36].

Conclusion

Carnivorous plants of *Nepenthes*, unlike other plant carnivores, offer the possibility of working with sterile digestive fluids as long as the pitchers are closed or the open pitchers kept somewhat sterile by means of gauze. Here, a novel FRET-based method regarding *Nepenthes* plants was established, optimized and successfully applied by using the fluorogenic PFU-093 substrate for easy and rapid detection of protease activities in digestive fluids of *Nepenthes* species. The specificity of the substrate for aspartic proteases provides a means of unravelling the processes involved in prey digestion in carnivorous pitcher plants and possibly in other carnivorous plants. The ability to measure induced protease activity in pitcher fluids is much more reflecting the digestive process as quantifying transcripts of the corresponding genes.

Supporting Information

S1 File. Fig. A in S1 File. FRET-peptide,-method and putative cleavage sites. **a** Simplified structure of the artificial substrate PFU-093, according to Kaman et al. [19]. The substrate contains a fluorescein isothiocyanate (FITC) as fluorophore (F) and Lysin-Dabcyl (LysDbc) as its quencher (Q) connected by a two valine (Val) bridge. The close vicinity of Q to F quenches the fluorescence. **b** Putative proteolytic cleavage sites of PFU-093 substrate either between the two Val or between the last Val and the LysDbc, resulting in cleavage products: 1) FITC-Val, 2) Val-LysDbc, 3) LysDbc; **c** predicted molecular masses of these products: 1) 619.68 g/mol, 2)

496.28 g/mol and 3) 397.47 g/mol. **Fig. B in S1 File. Substrate/product fluorescence dependence on different pH values. a** Pitcher fluid was incubated with PFU-093 substrate and pure water at 42°C for 10 h. 50 µl each of this mixture were given in different wells and mixed with additional 50 µl of 30 mM buffer solutions to adjust the final pH (2, 4, 6, 8, 10, black dots), before fluorescence was measured. Control with water instead of buffer was included, representing the original fluorescence. Arrows indicate the pH-depending change of fluorescence. **b** Digestive fluid and PFU-093 substrate were incubated in 1 mM citrate buffer pH 4, for 10 h at 42°C. Subsequently, the mixture was split up and pH was adjusted by topping with either 30 mM phosphate buffer, pH 8, (dark grey bar) or 30 mM citrate buffer, pH 4, (striped) before fluorescence measurement. **Fig. C in S1 File. *N. mirabilis* nepenthesin I, II (*Nm*NepI/ II) protein alignment compared to *N. gracilis* (*Ng*) and *N. alata* (*Na*) nepenthesin amino acid sequences**. The four levels of shading used are: *blue* > 80% sequence identity, *mid-blue* > 60% identity, *light blue* > 40% identity and *no shading* < 40%. Regions of predicted signal peptides and propeptides are named and the endings marked by a black stroke. Aspartic acid residues of the active center are indicated by a small black box and the flap tyrosine residue by a small green box, both above the sequences. The cysteine residues are represented through the colors: yellow, orange, green, light green, red and light red. The colored pairing of the residues show the disulphide bond arrangements in the primary structures of nepenthesin. **Fig. D in S1 File. Western blot of recombinant *N. mirabilis* nepenthesin II.** *Nm*NepII/Sf9 was expressed in Sf9 insect cell line, using an anti-V5 horseradish peroxidase antibody and ECL for detection Lanes represent 1) lysate, negative control, 2) culture medium, negative control, 3) lysate, positive control, 4) culture medium, positive control (CAT, catalase of 34 kDa); the blot also contains duplicates (clone 1 and 2) shown by the lanes 5) *Nm*NepII/Sf9, clone 1, lysate, 6) *Nm*NepII/Sf9, clone 1, culture medium, 7) *Nm*NepII/Sf9, clone 2, lysate, 8) *Nm*NepII/Sf9, clone 2, culture medium, all in comparison to the Sf9 cell line stably expressing *Nm*NepII/Sf9, pointed out with 5 µl (lane 9) and 10 µl (lane 10), respectively. **Table A in S1 File. List of primer sequences used in this study**.
(PDF)

Acknowledgments

We thank Birgit Arnold, Tamara Krügel and the greenhouse team for growing *Nepenthes* plants. We also thank Dr. Yannick Pauchet for providing us insect cells and the expression vector. Gerhard Pauls, Dr. Anne Morgenstern and Dr. Stephan H. von Reuß we thank for help with HPLC- MS method and last but not least Wilhelm Boland and the Max Planck Society for continuous support. We also thank Emily Wheeler for editorial assistance

Author Contributions

Conceived and designed the experiments: FB AM. Performed the experiments: FB AY. Analyzed the data: FB AY AM. Contributed reagents/materials/analysis tools: WEK FJB. Wrote the paper: FB WEK FJB AY AM.

References

1. Darwin C (1875) Insectivorous Plants. London, UK: John Murray.
2. Heslop-Harrison Y (1975) Enzyme release in carnivorous plants. In: Dingle JT, Dean RT, editors. Lysozymes in biology and pathology, pp 525–578.
3. Juniper BE, Robins RJ, Joel DM (1989) The carnivorous plants. London, UK, Academic press.

4. Buch F, Rott M, Rottloff S, Paetz C, Hilke I, Raessler M, et al. (2013) Secreted pitfall-trap fluid of carnivorous Nepenthes plants is unsuitable for microbial growth. Ann Bot 111: 375–383. doi: 10.1093/aob/mcs287 PMID: 23264234
5. Eilenberg H, Pnini-Cohen S, Schuster S, Movtchan A, Zilberstein A (2006) Isolation and characterization of chitinase genes from pitchers of the plant *Nepenthes khasiana*. J Exp Bot 57: 2775–2784. PMID: 16829546
6. Rottloff S, Stieber R, Maischak H, Turini FG, Heubl G, Mithöfer A (2011) Functional characterization of a class III acid endochitinase from the traps of the carnivorous pitcher plant genus, Nepenthes. J Exp Bot 62: 4639–4647. doi: 10.1093/jxb/err173 PMID: 21633084
7. Mithöfer A (2011) Carnivorous pitcher plants: Insights in an old topic. Phytochemistry 72: 1678–1682. doi: 10.1016/j.phytochem.2010.11.024 PMID: 21185041
8. Renner T, Specht CD. (2013) Inside the trap: gland morphologies, digestive enzymes, and the evolution of plant carnivory in the Caryophyllales. Curr Opin Plant Biol 16: 436–42. doi: 10.1016/j.pbi.2013.06.009 PMID: 23830995
9. Vines SH (1897) The proteolytic enzyme of *Nepenthes*. Ann Bot 11: 563–584.
10. Amagase S, Nakayama S, Tsugita A (1969) Acid protease in Nepenthes. II. Study on the specificity of nepenthesin. J Biochem 66: 431–439. PMID: 5354017
11. Jentsch J (1972) Enzymes from carnivorous plants (*Nepenthes*). Isolation of the protease nepenthacin. FEBS Lett 21: 273–276. PMID: 11946525
12. Tökés ZA, Woon WC, Chambers SM (1974) Digestive enzymes secreted by the carnivorous plant *Nepenthes macferlanei L*. Planta 119: 39–46. doi: 10.1007/BF00390820 PMID: 24442407
13. An CL, Fukusaki E, Kobayashi A (2002) Aspartic proteinases are expressed in pitchers of the carnivorous plant *Nepenthes alata* Blanco. Planta 214: 661–667. PMID: 11882933
14. Athauda SBP, Matsumoto K, Rajapakshe S, Kuribayashi M., Kojima M, Kubomura-Yoshida N, et al. (2004) Enzymic and structural characterization of nepenthesin, a unique member of a novel subfamily of aspartic proteinases. J Biochem 381: 295–396. PMID: 15035659
15. Takahashi K, Athauda SBP, Matsumoto K, Rajapakshe S, Kuribayashi M, Kojima M, et al. (2005) Nepenthesin, a unique member of a novel subfamily of aspartic proteinases: enzymatic and structural characteristics. Curr Protein Peptide Sci 6: 513–525. PMID: 16381601
16. Takahashi K, Tanji M, Shibata C (2006) Variations in the content and isozymic composition of Nepenthesin in the pitcher fluids among *Nepenthes* species. Carniv Pl Newslett 36: 73–76.
17. Stephenson P and Hogan J (2006) Cloning and characterization of a ribonuclease, a cysteine proteinase, and an aspartic proteinase from pitchers of the carnivorous plant *Nepenthes ventricosa* Blanco. Int J Plant Sci 167: 239–248.
18. Kadek A, Tretyachenko V, Mrazek H, Ivanova L, Halada P, Rey M, et al. (2014) Expression and characterization of plant aspartic protease nepenthesin-1 from *Nepenthes gracilis*. Protein Expr Purif 95: 121–128. doi: 10.1016/j.pep.2013.12.005 PMID: 24365662
19. Kaman WE, Hulst AG, van Alphen PTW, Roffel S, van der Schans MJ, Merkel T, et al. (2011) Peptide-based fluorescence resonance energy transfer (FRET) protease substrates for the detection and diagnosis of *Bacillus* spp. J Anal Chem 83: 2511–2517. doi: 10.1021/ac102764v PMID: 21370823
20. Kaman WE, Voskamp-Visser I, de Jongh DM, Endtz HP, van Belkum A, Bikker FJ, et al. (2013) Evaluation of a D-amino-acid-containing fluorescence resonance energy transfer peptide library for profiling prokaryotic proteases. Anal Biochem 441: 38–43. doi: 10.1016/j.ab.2013.06.015 PMID: 23850560
21. Buch F, Pauchet Y, Rott M, Mithöfer A (2014) Characterization and heterologous expression of a PR-1 protein from traps of the carnivorous plant *Nepenthes mirabilis*. Phytochemistry 100: 43–50. doi: 10.1016/j.phytochem.2014.01.014 PMID: 24534104
22. Untergrasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. (2012) Primer3—new capabilities and interfaces. Nucleic Acids Res 40: e115. PMID: 22730293
23. Waterhouse AM., Procter JB., Martin DMA, Clamp M and Barton GJ (2009) "Jalview Version 2—a multiple sequence alignment editor and analysis workbench". Bioinformatics 25: 1189–1191. doi: 10.1093/bioinformatics/btp033 PMID: 19151095
24. Hepburn JS (1918) Biochemical studies of the pitcher liquor of *Nepenthes*. Proc Am Phil Soc 57: 112–129.
25. Lüttge U (1964) Untersuchungen zur Physiologie der Carnivoren- Drüsen. Planta 63: 103–117.
26. Nakayama S, Amagase S (1968) Acid protease in Nepenthes partial purification and properties of enzyme. Proc Jpn Acad 44: 358–362.

27. Nordahl Petersen T, Brunak S, von Heijne G and Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8: 785–786. doi: 10.1038/nmeth.1701 PMID: 21959131

28. Gupta R, Jung E, Brunak S (2004) Prediction of N-glycosylation sites in human proteins. N-glycosylation site predictor available at: http://www.cbs.dtu.dk/services/NetNGlyc/

29. Simões I, Faro C (2004) Structure and function of plant aspartic proteinases. Eur J Biochem 271: 2067–2075. PMID: 15153096

30. Runeberg-Roos P, Tormakangas K, Ostman A (1991) Primary structure of a barley-grain aspartic proteinase. A plant aspartic proteinase resembling mammalian cathepsin D. Eur J Biochem 202: 1021–1027. PMID: 1722454

31. Takahashi K, Suzuki T, Nishii W, Kubota K, Shibata C, Isobe T, et al. (2011) A cysteine endopeptidase ("Dionain") is involved in the digestive fluid of *Dionaea muscipula* (Venus's flytrap). Biosci Biotech Biochem 75: 346–348. PMID: 21307583

32. Schulze WX, Sanggaard KW, Kreuzer I, Knudsen AD, Bemm F, Thøgersen IB, et al. (2012)The protein composition of the digestive fluid from the Venus flytrap sheds lighton prey digestion mechanisms. Mol Cell Proteom 11: 1306–1319.

33. Rottloff S, Müller U, Kilper R, Mithöfer A (2009) Micropreparation of single secretory glands from the carnivorous plant Nepenthes. Anal Biochem 394: 135–137. doi: 10.1016/j.ab.2009.07.013 PMID: 19602419

34. Pavlovič A, Slováková L, Šantrůček J (2011) Nutritional benefit from leaf litter utilization in the pitcher plant *Nepenthes ampullaria*. Plant Cell Environ 34: 1865–1873. doi: 10.1111/j.1365-3040.2011.02382.x PMID: 21707655

35. Nakamura Y, Reichelt M, Mayer VE, Mithöfer A (2013) Jasmonates trigger prey-induced formation of outer stomach in carnivorous sundew plants. Proc R Soc B 280: 20130228. doi: 10.1098/rspb.2013.0228 PMID: 23516244

36. Mithöfer A, Reichelt M, Nakamura Y (2014) Wound and insect-induced jasmonate accumulation in carnivorous *Drosera capensis*: two sides of the same coin. Plant Biol 16: 982–987 doi: 10.1111/plb.12148 PMID: 24499476

37. Escalante-Perez M, Krol E, Stange A, Geiger D, Al-Rasheid KA, Hause B, et al. (2011) A special pair of phytohormones controls excitability, slow closure, and external stomach formation in the Venus flytrap. PNAS USA 108: 15492–15497 doi: 10.1073/pnas.1112535108 PMID: 21896747

38. Libiaková M, Floková K, Novák O, Slováková L and Pavlovič A (2014) Abundance of cysteine endopeptidase dionain in digestive fluid of Venus flytrap (*Dionaea muscipula* Ellis) is regulated by different stimuli from prey through jasmonates. PLoS ONE 9: e104424. doi: 10.1371/journal.pone.0104424 PMID: 25153528

3.2 Manuscript II

Annals of Botany **118**: 369–375, 2016
doi:10.1093/aob/mcw110, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

Slow food: insect prey and chitin induce phytohormone accumulation and gene expression in carnivorous *Nepenthes* plants

Ayufu Yilamujiang[1], Michael Reichelt[2] and Axel Mithöfer[1,*]

[1]Department of Bioorganic Chemistry and [2]Department of Biochemistry, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, 07745 Jena, Germany
**For correspondence. E-mail amithoefer@ice.mpg.de*

Received: 15 March 2016 Returned for revision: 25 April 2016 Accepted: 29 April 2016 Published electronically: 20 June 2016

• **Background and Aims** Carnivorous *Nepenthes* plants use modified leaves forming pitfall traps to capture and digest prey, mainly insects, for additional nutrient supply. These traps, so called pitchers, contain a plant-derived fluid composed of many hydrolytic enzymes and defence-related proteins. In this study, the prey-induced induction of corresponding genes of those proteins and a role for phytohormones in this process was analysed.
• **Methods** Tissue from insect prey-fed, chitin- and phytohormone-challenged pitchers was harvested and analysed for selected gene expressions by a quantitative PCR technique. Phytohormone levels were determined by LC-MS/MS. Nepenthesin proteolytic activities were measured in the digestive fluid using a fluorescence substrate.
• **Key Results** Insect prey in the pitchers induced the accumulation of phytohormones such as jasmonates as well as the transcription of studied genes encoding a chitinase 3 and a protease (nepenthesin I), whereas a defence-related protein (PR-1) gene was not induced. Treatment with chitin as a component of the insects' exoskeleton triggered the accumulation of jasmonates, the expression of nepenthesin I and chitinase 3 genes similar to jasmonic acid treatment, and induced protease activity in the fluid. All detectable responses were slowly induced.
• **Conclusions** The results suggest that upon insect prey catch a sequence of signals is initiated: (1) insect-derived chitin, (2) jasmonate as endogenous phytohormone signal, (3) the induction of digestive gene expression and (4) protein expression. This resembles a similar hierarchy of events as described from plant pathogen/herbivore interactions, supporting the idea that carnivory evolved from plant defences.

Key words: *Nepenthes*, insect prey, phytohormones, jasmonates, chitin, nepenthesin, carnivorous plant, gene induction.

INTRODUCTION

Carnivory in plants is an adaptation for nutrient-poor environments. Different species of carnivorous plants developed various strategies to obtain additional nutrients from caught insect prey, such as sticky flypaper traps in *Drosera* species or snap traps in *Dionaea muscipula* (Juniper *et al.*, 1989). Plants of the genus *Nepenthes* have so-called pitcher traps, representing metamorphosed leaves. Pitchers can be divided into three zones: at the top a peristome that is involved in attracting and trapping the prey; second a slippery waxy zone on the inner side of the pitchers that is involved in trapping and preventing prey escape; and finally, at the bottom the digestive zone which is covered inside with bifunctional glands and contains a digestive fluid. On the one hand, the bifunctional glands secrete hydrolytic enzymes into the fluid and on the other hand they take up the nutrients which are generated by prey digestion in the digestive fluid (Mithöfer, 2011).

There has been a long history of interest in the function and composition of digestive pitcher fluids and some pioneer suggestions about their hydrolytic properties were reported by Charles Darwin (Darwin, 1875). Various hydrolytic proteins have been identified meanwhile, for some of which their corresponding genes were cloned, heterologously expressed and further characterized (Mithöfer, 2011). The most prominent proteins in the pitcher fluid of *Nepenthes* are two pepsin-related aspartic proteases, nepenthesin I and II, which cleave peptides at the carboxyl side of aspartic acid residues. Nepenthesins were purified, cloned and biochemically characterized from different *Nepenthes* species (An *et al.*, 2002; Athauda *et al.*, 2004; Takahashi, 2007; Buch *et al.*, 2015). These proteins exhibit stable enzymatic activity up to 60 °C and low pH, which enables the use of nepenthesins for technical applications (Kadek *et al.*, 2014). Besides enzymes with hydrolytic activities, some proteins found in the pitcher fluids of *Nepenthes* are known to have antimicrobial properties, such as thaumatin-like protein (Rottloff *et al.*, 2009) or the pathogenesis-related protein-1 (PR-1) (Buch *et al.*, 2014). These two examples represent typical pathogenesis-related (PR) proteins with well-known function in plant defence reactions against microbial pathogen attack (van Loon *et al.*, 2006). Strikingly, many of the hydrolytic enzymes in the pitcher fluid are classified as PR proteins as well, for instance chitinases, proteases and glucanases, suggesting that carnivory in plants has evolved from existing defensive reactions.

In recent years, most studies on *Nepenthes* pitcher fluid have focused on elucidation of the protein composition and they contributed significantly to our knowledge and understanding of

prey digestion in these carnivorous plants. However, our knowledge of the molecular regulation of protein secretion in *Nepenthes* remains limited.

Given that most of the identified pitcher fluid proteins belong to one of the PR protein families (Mithöfer, 2011), it is tempting to speculate that their gene expressions might be initiated by signals derived from caught prey. Furthermore, the endogenous regulation might be mediated by defence-related phytohormones such as jasmonates [jasmonic acid, JA; jasmonic acid–isoleucin conjugate, JA-Ile; *cis*-(+)-12-oxophytodienoic acid, *cis*-OPDA] and salicylic acid (SA), similar to the situation in which PR-protein-encoding genes are up-regulated upon pathogen attack. Based on this hypothesis, in this study we investigated the induction of gene expression of selected PR proteins upon insect prey treatment and the role of related phytohormones.

MATERIAL AND METHODS

Plant material and treatment

Nepenthes alata Blanco plants were grown in growth chambers at 20–25 °C, 80–85 % humidity, with a 16-h light/8-h dark photoperiod. To avoid any contamination, 2-week-old and still closed pitchers were used for chitin and phytohormone injections as closed pitcher fluid is known to be free from microbes (Buch *et al*., 2013). One milligram of colloidal chitin in 500 μL water or only water as a control was injected into the pitcher using sterile pipette tips. For phytohormone-induced gene expression experiments, JA (200 μM final concentrations in the pitcher fluid) or water for controls was injected. For fruit fly (*Drosophila melanogaster*) treatment pitchers were covered with gauze when they were still closed. Covered pitchers were fed with wild-type *D. melanogaster* after 2 weeks of pitcher opening according to Buch *et al.* (2015). In all experiments where phytohormones or gene inductions were analysed in the pitcher tissue, an individual pitcher (biological replicate) was used for one measurement. At least three biological replicates, depending on the availability of utilizable pitchers, were taken for one data point. To analyse JA-Ile directly in the pitcher fluid, 100 μL of pitcher fluid was taken out for each time point (0, 2, 4, 6, 8, 10, 24, 30, 48, and 72 h) after fruit fly treatment. The removed volume was replaced with 25 mM KCl, according to Buch *et al*. (2013).

Expression analysis by real-time PCR

Tissue from the digestive zone of the pitcher was harvested and frozen in liquid nitrogen and stored in − 80 °C until used. For analysis, frozen plant material was homogenized for 40 s at 1200 r.p.m. in a Genogrinder 2010 (Spex Sample Prep, Stanmore, UK). Total RNA was isolated using an Invitrap Spin Plant RNA Mini kit (STRATEC Molecular, Berlin, Germany) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany) by using Oligo (dT)12–18 and according to the manufacturer's protocol. For expression analysis by real-time PCR, actin was used as a housekeeping gene by using the following primers: forward primer, 5′-CTCTTAACCCCAAAGCAAACAGG-3′; and reverse primer, 3′-GTGAGAGAACAGCCTGGATG-5′. *Nepenthes alata* aspartic protease nepenthesin, *Nep I* (forward primer, 5′-CG GGCAAAAACTTAACCAAA-3′; reverse primer, 3′-ATCCA TGATTGCGGAGAAAG-5′); *N. alata* class 3 chitinase, *Chit3* (forward primer, 5′-GCTCCGGCATAGCAGTCTAC-3′; and reverse primer, 3′-CTTGGTTTTGGCATGAGGTT-5′); *N. mirabilis* pathogenesis-related protein-1, *PR-1* (forward primer, 5′-GCCATTGGTATCATCCAACC-3′; and reverse primer, 3′-AGAGCATAAGCCGCAACAGT-5′). Real-time PCR for gene expression analysis was performed in 96-well plates on a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) by using Brilliant II QPCR SYBR green Mix (Agilent, Böblingen, Germany). In total, 60 ng cDNA was used for each sample with a total reaction volume of 25 μL. Obtained mRNA levels of the gene of interest were normalized with respect to the actin mRNA level of each sample, according to Rottloff *et al*. (2011). Calculations of expression ratios of target genes were related to the mRNA level of target genes in control tissue, which were defined as 1·0; this calculation was done according to Pfaffl (2001). To ensure that gene expression levels were comparable at all different time points, the particular Δct values for the controls of each target gene related to actin gene expression were determined. Only sets of experiments with an s.d. of ≤ 5 % were used for further calculations. All assays were run at least in independent triplicates (biological replications), each biological replicate consisting of three technical replicates.

Nepenthesin protease activity measurement in pitcher fluid

Proteolytic nepenthesin activity in the pitcher fluid was measured by using the specific fluorescence resonance energy transfer-based substrate PFU-093, as described (Kaman *et al*., 2011; Buch *et al*., 2015). Briefly, 50 μL of control or induced pitcher fluid, 39 μL water and 1 μL of 80 μM fluorescence substrate PFU-093 were mixed in black 96-well microtitre plates (Greiner Bio-one GmbH, Frickenhausen, Germany). Plates were incubated for 5 h at room temperature. After incubation, 10 μL of 100 mM Tris-HCl buffer, pH 8·5, was added to the mixture before measurement. Fluorescence of cleaved substrate was measured, at 42 °C using a microplate reader (Tecan infinite M200, Männedorf, Switzerland) with excitation/emission wavelength of 485/530 nm, respectively.

Quantification of phytohormones

Phytohormone analyses were carried out according to Vadassery *et al*. (2012) with some modifications. Thus, harvested plant material (digestive zone of the pitcher) was frozen with liquid nitrogen and stored at − 80 °C until used. For analysis plant material was finely ground in liquid nitrogen and weighed. Ground plant material (250 mg) was extracted with 1·5 mL of methanol containing internal standard: 60 ng of D_6-jasmonic acid (HPC Standards GmbH, Cunnersdorf, Germany), 60 ng D_4-salicylic acid (Sigma-Aldrich, St Louis, MO, USA) and 12 ng of JA–$^{13}C_6$-isoleucine conjugate which was synthesized as described by Kramell *et al*. (1988). The homogenate was mixed for 30 min, centrifuged at 14000 r.p.m. for 10 min at

4 °C, subsequently re-extracted with 500 µL methanol and both supernatants were pooled. The combined extract was dried, re-dissolved in 500 µL methanol and used for chromatography. For analysis of pitcher fluid, the fluid was spiked with 2 ng mL^{-1} of fluid for JA–$^{13}C_6$-isoleucine conjugate and used for chromatography. Separation was performed on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Zorbax Eclipse XDB-C18 column (50 × 4·6 mm, 1·8 µm; Agilent). Formic acid (0·05 %) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0–0·5 min, 5 % B; 0·5–9·5 min, 5–42 % B; 9·5–9·51 min, 42–100 % B; 9·51–12 min, 100 % B; and 12·1–15 min, 5 % B keeping a flow rate of 1·1 mL min^{-1}. Column temperature was maintained at 25 °C. An API 5000 tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turbospray ion source was operated in negative ionization mode. Ion spray voltage was maintained at − 4500 eV, turbo gas temperature was set at 700 °C, nebulizing gas was set at 60 p.s.i., curtain gas at 25 p.s.i., heating gas at 60 p.s.i. and collision gas at 7 p.s.i. To monitor analyte parent ion → product ion, multiple reaction monitoring (MRM) was used: *m/z* 209·1 →59·0 (collision energy (CE) − 24 V; declustering potential (DP) − 35 V) for JA; *m/z* 215·1 → 59·0 (CE − 24 V; DP − 35 V) for D_6-JA; *m/z* 136·9 → 93·0 (CE − 22 V; DP − 35 V) for SA; *m/z* 140·9 → 97·0 (CE − 22 V; DP − 35 V) for D_4-SA; *m/z* 290·9 → 165·1 (CE − 24 V; DP − 45 V) for *cis*-OPDA, *m/z* 322·2 → 130·1 (CE − 30 V; DP − 50 V) for JA-Ile conjugate; *m/z* 328·2 → 136·1 (CE − 30 V; DP − 50 V) for JA–$^{13}C_6$-Ile conjugate. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Phytohormones were quantified relative to the signal of their corresponding internal standard. For quantification of *cis*-OPDA, D_6-JA was used as the internal standard applying an experimentally determined response factor of 0·5.

RESULTS AND DISCUSSION

Fruit-fly-induced PR gene expressions and phytohormone accumulation

Carnivorous plants of the genus *Nepenthes* digest insect prey in the pitcher trap fluid to supplement their nutrient demand. The pitcher fluid is present in the bottom part of the trap and its composition is supplied by secretion processes via specific glands. To study insect prey-induced gene expression in the pitcher tissue, three corresponding genes of well-characterized proteins from the pitcher fluid were chosen, namely nepenthesin I (Nep I; Buch *et al.*, 2015), class III chitinase (Chit3; Hatano and Hamada, 2012) and pathogenesis-related protein 1 (PR-1; Buch *et al.*, 2014). Gene expression of the aspartic protease *Nep I* was slightly but significantly upregulated after 12 and 24 h and strongly after 48 h of fruit fly treatment compared with the control. *Chit3* was also slightly upregulated after 24 h and strongly after 48 h. In contrast, *PR-1* gene expression did not change (Fig. 1). Because fruit flies represent insect prey, the result obtained can be explained by the fact that both upregulated genes represent enzymes involved in prey digestion. PR-1, by contrast, has no enzymatic but antimicrobial activity (Buch *et al.*, 2014), which might not be necessary to establish

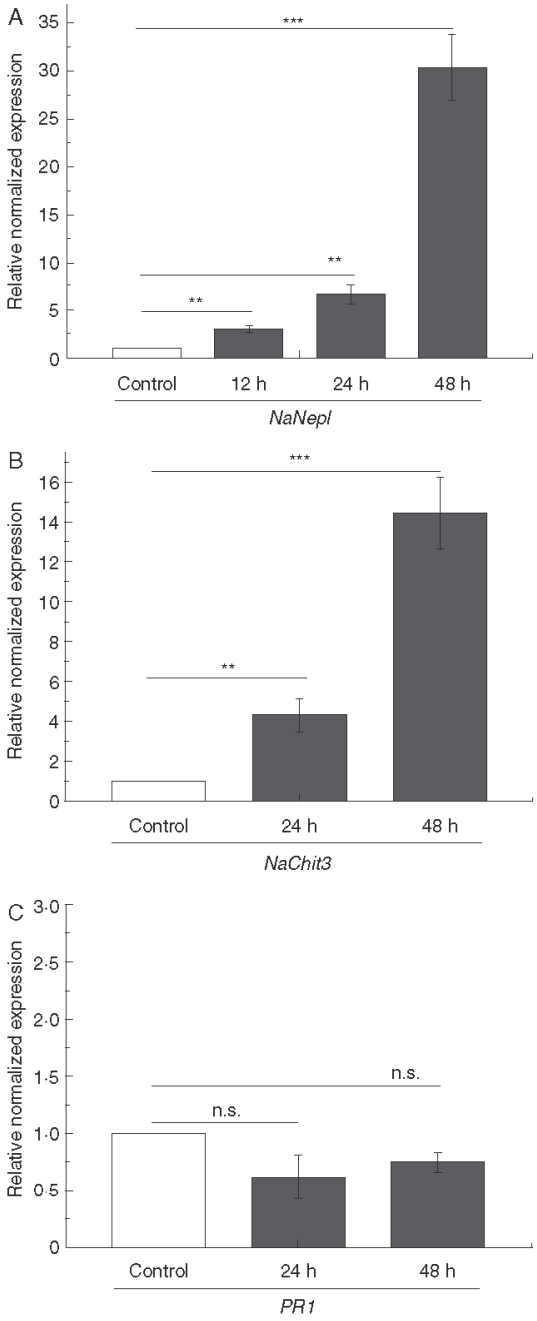


FIG. 1. Fruit fly-induced PR gene expression in *N. alata* pitchers. Pitchers were fed with 60 mg fruit flies and mean expression (± s.e.; $n = 3$–5) of selected PR genes was analysed. *NepI* (A) expression was analysed after 12, 24 and 48 h; *Chit3* (B) and *PR-1* (C) were analysed after 24 and 48 h both in untreated (white) and treated (grey) pitchers. Statistically significant differences between control and treated pitchers were analysed by Student's *t*-test for each gene separately. Gene expression for control was set as 1·0; n.s.: not significant, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

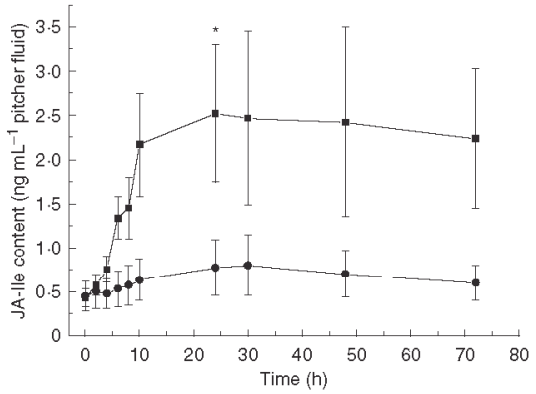


FIG. 2. JA-Ile accumulation in fruit fly-fed pitcher fluids of *N. alata*. Pitchers were fed with 60 mg fruit flies and the level of JA-Ile was analysed after addition of fruit flies at different time points in pitcher fluids. Mean JA-Ile content (± s.e.) was analysed in both control (•) ($n = 3$) and fruit fly-treated (■) ($n = 7$) pitchers. Statistically significant differences between control and treated pitchers were analysed for each time point by Student's *t*-test separately; *$P < 0{\cdot}05$.

at this time point. Furthermore, the result found for *Nep I* corresponds well to a result that was recently published (Buch *et al.*, 2015), in which the authors showed that after 48 h the enzymatic nepenthesin I activity in the pitcher fluid was strongly increased when fed with fruit flies. In the same study, they showed that nepenthesin I activity was induced by JA treatment alone, but only after several days.

Recently it has been suggested for different carnivorous plants that phytohormones are involved in the digestion process, for example in *Drosera capensis* (Nakamura *et al.*, 2013) or Venus flytrap (*Dionaea muscipula*). In the latter case the application of JA induced the abundance of a cysteine protease, dionain, and increased proteolytic activity in the digestive fluid (Libiakova *et al.*, 2014). Escalante-Perez *et al.* (2011) described the involvement of *cis*-OPDA in the induction of digestive fluid secretion in *D. muscipula*. Those results also strongly suggest a role for phytohormones in the fruit fly-induced process of gene inductions in *Nepenthes*. To prove this hypothesis, the level of the biologically most active jasmonate, JA-Ile, was first determined in the pitcher fluid of *N. alata* at various time points after *D. melanogaster* treatment (Fig. 2). Such an experiment was necessary to gain insight into the kinetics of jasmonate accumulation induced by caught prey. As shown in Fig. 2, when insect prey was added, JA-Ile concentration increased continuously up to 24 h and stayed at that level, indicating a relative slow phytohormone response. One advantage of this experiment was that the samples could be harvested continuously from the pitchers, showing the dynamics of the system; a disadvantage was that phytohormones are generated and active in the plant tissue. However, using one pitcher for one measurement for several biological replicates and numerous time points is simply not feasible as the number of developmentally comparable pitchers is limited. Therefore, the results obtained from the pitcher fluid experiment provided valuable data and the basis for subsequent experiments for the analysis of different phytohormones in the tissue at defined time points. After fruit fly treatment, jasmonates, i.e. JA and JA-Ile as well as their biosynthetic precursor, *cis*-OPDA, significantly accumulated over time in pitcher tissue (Fig. 3). Also, the SA level was found to be significantly higher after 48 h of fruit fly treatment [0 h: 2·3 ± 0·5 ng SA (g f. wt)$^{-1}$; 48 h: 8·5 ± 1·9 ng SA (g f. wt)$^{-1}$; $P < 0{\cdot}005$, Student's *t*-test]. This indicates that prey catch and the subsequently digestion in *Nepenthes* is very likely a phytohormone-mediated process, depending at least on jasmonates.

Chitin-induced PR gene expression and phytohormone accumulation

To further investigate whether a certain signal derived from insect prey might be sufficient to induce gene expression and phytohormone accumulation in *Nepenthes*, chitin as a major component of the insects' exoskeleton was chosen to mimic caught prey. It has already been reported that in *N. khasiana* pitchers chitin can induce the accumulation of naphthoquinones (Eilenberg *et al.*, 2010; Raj *et al.*, 2011) as well as gene expression of chitinases, e.g. type *NkChit1b* (Eilenberg *et al.*, 2006; Hatano and Hamada, 2012). Interestingly, compared with the non-treated control, chitin-induced *Nep I* gene expression was determined after 24 h; for *Chit3* and *PR-1* only a trend in the same direction was detected (Fig. 4). After 48 h the situation was different. *Nep I* gene expression was downregulated to control level but *Chit3* transcripts were found to be upregulated; again, *PR-1* showed no obvious differences compared with the non-treated control pitchers (Fig. 4). For *PR-1* and *Chit3* the situation after 48 h resembles the situation found after fruit fly treatment (Fig. 1), suggesting that chitin very likely represents at least one of probably more insect prey-derived signalling compounds that is involved in the induction of downstream events in *Nepenthes* necessary to initiate the digestion process. However, for *Nep I* the situation is different. Here, after 48 h challenge with chitin, gene expression is already down again whereas upon fruit fly treatment a high level of transcripts was found. This situation can be explained by the fact that after chitin application this particular signal molecule is present in relatively high concentrations, can act directly and must not be released from the prey. Therefore, it might act faster. Moreover, the much higher induction of *Nep I* after 48 h upon challenge with *D. melanogaster* (Fig. 1A) could be due to additional signals that are absent upon sole chitin treatment but might be released slowly from the whole insect prey. Interestingly, when the pitchers were treated with JA (200 μM), a weak *Nep I* gene induction (1·5-fold; $P < 0{\cdot}05$, Student's *t*-test) was determined only after 24 h, whereas the *Chit3* gene was found to be induced (1·6-fold; $P < 0{\cdot}05$, Student's *t*-test) only after 48 h, comparable to the induction pattern upon chitin treatment. Together, these results suggest different gene regulation mechanisms among the three selected genes.

Based on the former results it was tempting to speculate that chitin can also induce phytohormone accumulation. This was investigated again in comparison with non-treated control pitchers. After 24 h, JA and *cis*-OPDA only showed a trend to higher concentrations but after 48 h the level of both compounds was significantly enhanced. JA-Ile showed significantly higher concentrations after 24 h and to the same extent after 48 h (Fig. 5B). Strikingly, the basic concentrations of *cis*-OPDA and JA-Ile, in the latter case less pronounced, were about 1·5- to

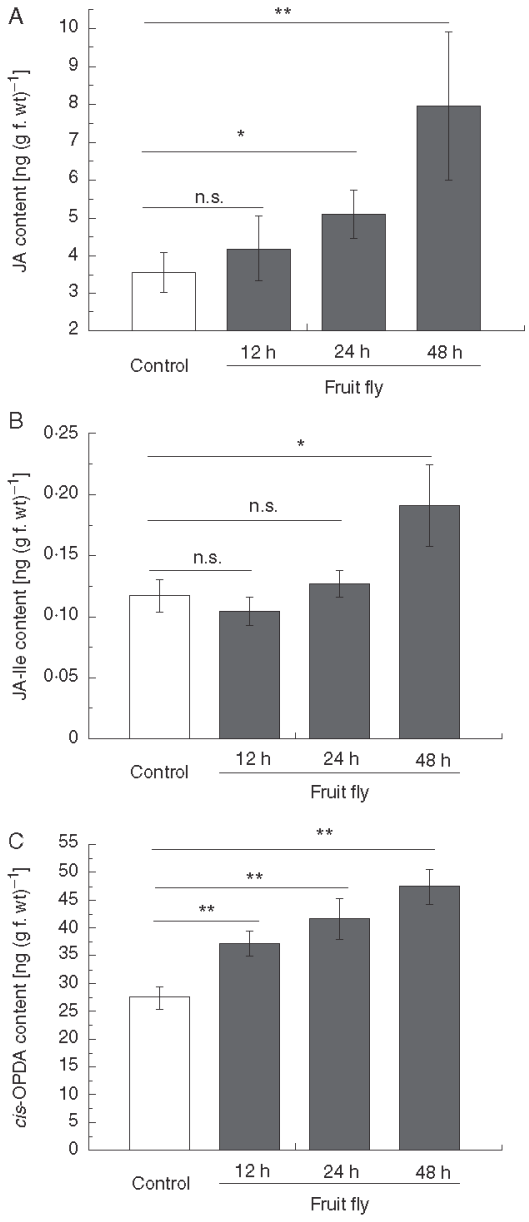


FIG. 3. Phytohormone accumulation in fruit fly-fed pitchers of *N. alata*. Pitchers were fed with 60 mg fruit flies and endogenous phytohormone levels of JA (A), JA-Ile (B) and *cis*-OPDA (C) were analysed after 12, 24 and 48 h in pitcher tissue. Mean phytohormone content (± s.e.; $n = 3$–5) was analysed in both untreated control (white) and treated (grey) pitchers. Statistically significant differences between control and treated pitchers were analysed by Student's *t*-test for each hormone separately; n.s.: not significant, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

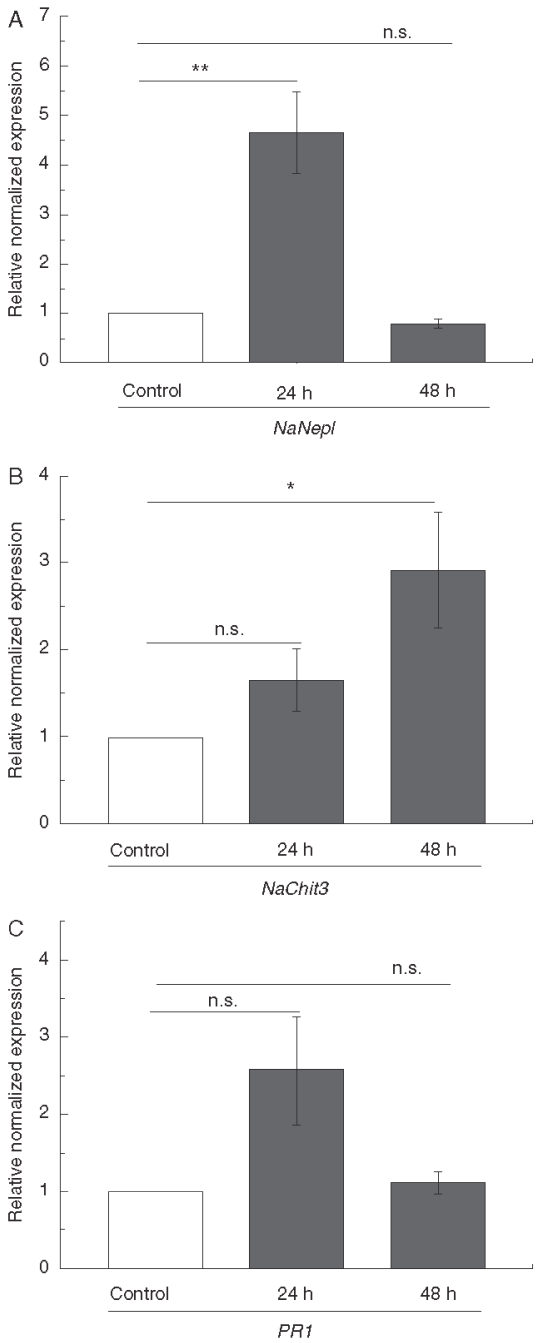


FIG. 4. Chitin-induced PR gene expression in pitcher tissue of *N. alata*. Pitchers were treated with chitin and mean expression (±s.e.; $n = 3$) of selected PR genes *NepI* (A), *Chit3* (B) and *PR-1* (C) was analysed after 24 and 48 h in both untreated (white) and treated (grey) pitchers. Statistically significant differences between control and treated pitchers were analysed by Student's *t*-test for each gene separately. Gene expression for control was set as 1·0; n.s.: not significant, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

2-fold higher in the experiments with chitin treatment (Fig. 5) compared with the fruit fly experiments (Fig. 3). This is probably due to slightly different developmental stages of the pitchers. Whereas the pitchers for fruit fly feeding need to be open,

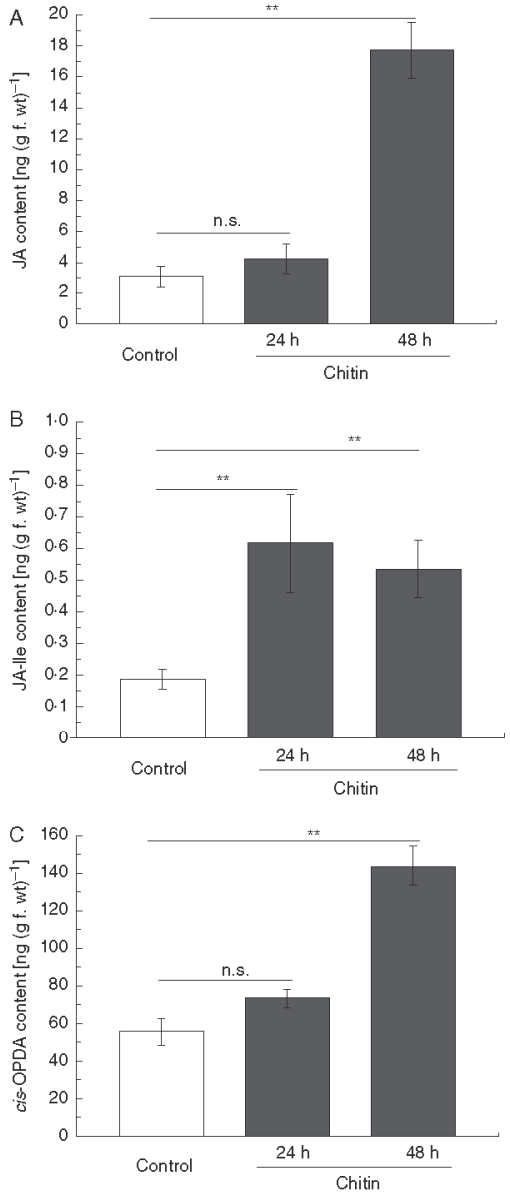


FIG. 5. Jasmonate induction in chitin-treated pitcher tissue of *N. alata*. Pitchers were treated with chitin and endogenous phytohormone levels of JA (A), JA-Ile (B) and *cis*-OPDA (C) were analysed after 24 and 48 h in pitcher tissue. Mean phytohormone content (±s.e.; $n = 3$–5) was analysed in both control (white) and treated (grey) pitchers. Statistically significant differences between control and treated pitchers were analysed by Student's *t*-test separately; n.s.: not significant, $^{**}P < 0{\cdot}01$.

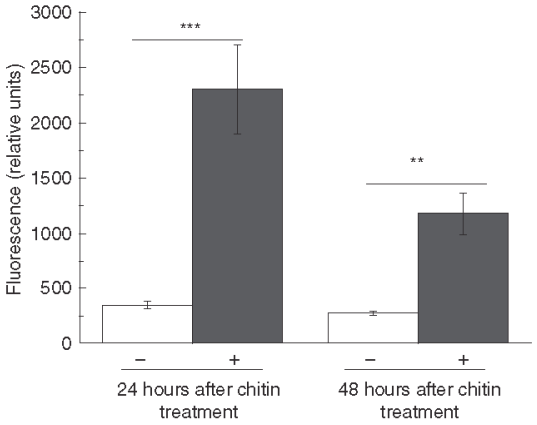


FIG. 6. Chitin-induced nepenthesin activity in the pitcher fluid of *N. alata*. Pitchers were treated with chitin and nepenthesin protease activity in the pitcher fluid was measured by using a fluorescence substrate (PFU-093) at 24 and 48 h after treatment. Mean relative fluorescence (±s.e.; $n = 3$–4) was analysed in both control (white) and treated (grey) pitchers. Statistically significant differences between control and treated pitchers were analysed by Student's *t*-test; $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

for chitin treatment unopen pitchers were used to avoid any contamination. However, the corresponding controls were always performed to see the difference between non-treated and treated pitchers. SA concentrations did not show any changes after 24 h [0 h: 3·8 ± 0·6 ng SA (g f. wt)$^{-1}$; 24 h: 3·8 ± 0·7 ng SA (g f. wt)$^{-1}$; not significant, Student's *t*-test] but were found nearly doubled after 2 d of chitin treatment (48 h: 7·1 ± 0·8 ng SA (g f. wt)$^{-1}$; $P < 0{\cdot}01$, Student's *t*-test).

Chitin-induced nepenthesin activity

Gene expression analyses provided evidence that the encoded proteins and corresponding enzymatic activities are probably upregulated as well; in fact, however, this had to be proven. Thus, the proteolytic activity of Nep 1 was exemplarily measured in the digestive fluid upon chitin treatment of the pitchers. Indeed, after 24 and 48 h, significantly higher enzymatic activities were detected compared with the non-treated control pitchers (Fig. 6). Strikingly, again establishment of the enzymatic activity was faster upon chitin than fruit fly treatment (Buch *et al.*, 2015), resembling and supporting the results obtained for gene expression.

CONCLUSIONS

The results presented here support the hypothesis that plant carnivory evolved from plant–herbivore interactions, in particular employing elements of plant defence against herbivorous insects. This includes jasmonates as endogenous signalling molecules, such as JA and JA-Ile, as well as preceding recognition of a prey-related signal. In the case of caught insects in *Nepenthes*, chitin represents such a signal. The prey-initiated signalling cascade eventually leads to the induction of genes encoding enzymes involved in prey digestion but also classified as PR proteins, participating in plant defence. Thus, another tessera is provided suggesting that the origin of carnivory in plants developed from plant defences. Strikingly, insect-induced phytohormone accumulations, gene expression and enzymatic

activities are detectable only with delay, if compared with insect attack-induced defence responses. This suggests that the onset of digestion in carnivorous *Nepenthes* plants is a comparatively slow process, perhaps because the prey is already caught and there is no need for the plant to hurry.

ACKNOWLEDGEMENTS

We thank Birgit Arnold and the whole greenhouse team at the MPI for Chemical Ecology for growing *Nepenthes* plants; Floris J. Bikker, Amsterdam, for providing PFU-093 substrate; Silke Trautheim for providing fruit flies; Sandra Scholz for continuous help; and Wilhelm Boland and the Max Planck Society for support.

LITERATURE CITED

An CI, Fukusaki E, Kobayashi A. 2002. Aspartic proteinases are expressed in pitchers of the carnivorous plant *Nepenthes alata* Blanco. *Planta* **214**: 661–667.

Athauda SBP, Matsumoto K, Rajapakshe S, *et al.* 2004. Enzymic and structural characterization of nepenthesin, a unique member of a novel subfamily of aspartic proteinases. *Biochemical Journal* **381**: 295–306.

Buch F, Rott M, Rottloff S, *et al.* 2013. Secreted pitfall-trap fluid of carnivorous *Nepenthes* plants is unsuitable for microbial growth. *Annals of Botany* **111**: 375–383.

Buch F, Pauchet Y, Rott M, Mithöfer A. 2014. Characterization and heterologous expression of a PR-1 protein from traps of the carnivorous plant *Nepenthes mirabilis*. *Phytochemistry* **100**: 43–50.

Buch F, Kaman WE, Bikker FJ, Yilamujiang A, Mithöfer A. 2015. Nepenthesin protease activity indicates digestive fluid dynamics in carnivorous *Nepenthes* plants. *PloS One* **10**: e0118853.

Darwin C. 1875. *Insectivorous plants*. London: John Murray.

Eilenberg H, Pnini-Cohen S, Schuster S, Movtchan A, Zilberstein A. 2006. Isolation and characterization of chitinase genes from pitchers of the carnivorous plant *Nepenthes khasiana*. *Journal of Experimental Botany* **57**: 2775–2784.

Eilenberg H, Pnini-Cohen S, Rahamim Y, *et al.* 2010. Induced production of antifungal naphthoquinones in the pitchers of the carnivorous plant *Nepenthes khasiana*. *Journal of Experimental Botany* **61**: 911–922.

Escalante-Perez M, Krol E, Stange A, *et al.* 2011. A special pair of phytohormones controls excitability, slow closure, and external stomach formation in the Venus flytrap. In: *Proceedings of the National Academy of Sciences of the United States of America* **108**: 15492–15497.

Hatano N, Hamada T. 2012. Proteomic analysis of secreted protein induced by a component of prey in pitcher fluid of the carnivorous plant *Nepenthes alata*. *Journal of Proteomics* **75**: 4844–4852.

Juniper BE, Robins RJ, Joel DM. 1989. *The carnivorous plants*. San Diego, CA: Academic Press.

Kadek A, Mrazek H, Halada P, Rey M, Schriemer DC, Man P. 2014. Aspartic protease nepenthesin-1 as a tool for digestion in hydrogen/deuterium exchange mass spectrometry. *Analytical Chemistry* **86**: 4287–4294.

Kaman WE, Hulst AG, van Alphen PTW, *et al.* 2011. Peptide-based fluorescence resonance energy transfer protease substrates for the detection and diagnosis of *Bacillus* species. *Analytical Chemistry* **83**: 2511–2517.

Kramell R, Schmidt J, Schneider G, Sembdner G, Schreiber K. 1988. Synthesis of n-(jasmonoyl)amino acid conjugates. *Tetrahedron* **44**: 5791–5807.

Libiakova M, Flokova K, Novak O, Slovakova L, Pavlovic A. 2014. Abundance of cysteine endopeptidase dionain in digestive fluid of Venus flytrap (*Dionaea muscipula* Ellis) is regulated by different stimuli from prey through jasmonates. *PLoS One* **9**: e104424.

Mithöfer A. 2011. Carnivorous pitcher plants: insights in an old topic. *Phytochemistry*, **72**: 1678–1682.

Nakamura Y, Reichelt M, Mayer VE, Mithöfer A. 2013. Jasmonates trigger prey-induced formation of ‚outer stomach' in carnivorous sundew plants. *Proceedings of the Royal Society B – Biological Sciences* **280**: 20130228

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acid Research* **29**: e45.

Raj G, Kurup R, Hussain AA, Baby S. 2011. Distribution of naphthoquinones, plumbagin, droserone, and 5-*O*-methyl droserone in chitin-induced and uninduced *Nepenthes khasiana*: molecular events in prey capture. *Journal of Experimental Botany* **62**: 5429–5436.

Rottloff S, Müller U, Kilper R, Mithöfer A. 2009. Micropreparation of single secretory glands from the carnivorous plant *Nepenthes*. *Analytical Biochemistry* **394**: 135–137.

Rottloff S, Stieber R, Maischak H, Turini FG, Heubl G, Mithöfer A. 2011. Functional characterization of a class III acid endochitinase from the traps of the carnivorous pitcher plant genus, *Nepenthes*. *Journal of Experimental Botany* **62**: 4639–4647.

Takahashi K. 2007. Variations in the content and isozymic composition of nepenthesin in the pitcher fluids among *Nepenthes* species. *Carnivorous Plant Newsletter* **36**: 73–76.

Vadassery J, Scholz SS, Mithöfer A. 2012. Multiple calmodulin-like proteins in Arabidopsis are induced by insect-derived (*Spodoptera littoralis*) oral secretion. *Plant Signaling & Behavior* **7**: 1277–1280.

van Loon LC, Rep M, Pieterse CM. 2006. Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology* **44**: 135–162.

3.3 Manuscript III

Coprophagous features in carnivorous *Nepenthes* plants: a task for ureases

Ayufu Yilamujiang[1], Anting Zhu[2], Rodrigo Ligabue-Braun[3], Stefan Bartram[1], Claus-Peter Witte[2], Rainer Hedrich[4], Mitsuyasu Hasabe[5], Caroline R. Schöner[6], Michael G. Schöner[6], Gerald Kerth[6], Célia R. Carlini[3,7], Axel Mithöfer[1,*]

[1]Department of Bioorganic Chemistry, Max Plank Institute for Chemical Ecology, 07745 Jena, Germany.

[2]Institute of Plant Nutrition, Leibniz University Hannover, 30419 Hannover, Germany.

[3]Center of Biotechnology, Universidade Federal do Rio Grande do Sul, 91501-970 Porto Alegre, Brazil.

[4]Institute for Molecular Plant Physiology and Biophysics, University of Würzburg, 97082 Würzburg, Germany.

[5]National Institute for Basic Biology, Okazaki 444-8585, Japan.

[6]Zoological Institute and Museum, Ernst-Moritz-Arndt-Universität Greifswald, 17489 Greifswald, Germany.

[7]Brain Institute (BRAINS-InsCer), Pontifícia Universidade Católica do Rio Grande do Sul, 90610-000 Porto Alegre, Brazil.

*Correspondence to: A.M.

Axel Mithöfer; MPI for Chemical Ecology; Hans-Knöll-Str 8; D-07745 Jena, Germany

Tel: +49 3641 571263

Email: amithoefer@ice.mpg.de

Abstract

Most terrestrial carnivorous plants are specialized on insect prey digestion to obtain additional nutrients. Few species of the genus *Nepenthes* developed mutualistic relationships with mammals for nitrogen supplementation. In a cooperative interaction between *N. hemsleyana* and a bat, *Kerivoula hardwickii*, plant pitchers are used as roost, thereby the bat's excrement serves as nitrogen source, very likely by providing urea. However, enzymatic hydrolysis is necessary to further metabolize and use urea. Here, we show the presence and enzymatic activity of a urease for *Nepenthes* plant tissues. With ^{15}N-enriched urea provided to *Nepenthes* pitchers we demonstrate that urea is metabolized and its nitrogen is distributed within the plant. The corresponding

urease cDNA from *N. hemsleyana* was isolated and functionally expressed. A comprehensive phylogenetic analysis for eukaryotic ureases, including *Nepenthes* and five other carnivorous plants' taxa, identified them as canonical ureases and reflects the plant phylogeny. Hence, this study reveals ureases as an emblematic example for an efficient but high adaptive plasticity in plants while developing a further specialized lifestyle from carnivory to coprophagy.

A striking feature of plants is the ability to adapt with high flexibility to completely different ecological environments and to survive even in extreme habitats. Some plants that live in nutrient poor environments evolved carnivory to obtain nitrogen, phosphorus and minerals from animals. Various trapping mechanisms exist in carnivorous plants such as pitfall traps (*Nepenthes* spp.), adhesive traps (*Drosera* spp.), snap traps (*Dionaea muscipula*), corkscrew traps (*Genlisea* spp.), and suction traps (*Utricularia* spp.) supporting a broad spectrum of prey selection[1]. In all cases, the different traps derived from metamorphosis of leaves forming new adaptive organs[1]. Carnivorous plants attracted attention of scientists for centuries, including Charles Darwin, who already in 1875 reported pioneer suggestions on plant carnivory[2]. In recent years, proteomic and molecular approaches provided many new insights into plant carnivory[3, 4, 5, 6, 7, 8]. The more we learn about carnivory in plants the more we realize that its basis relies on the plants' ability to defend themselves against attackers such as herbivorous insects or microbes. In a simplified view we may state that carnivorous plants use already existing pathways and strategies, from signaling to hydrolytic, defense-related enzymes, and transferred these mechanisms into a different ecological context, i.e. carnivory (e.g. in *Dionaea*[3]; *Drosera*[6, 7]; *Nepenthes*[5, 8], *Cephalotus*[4]).

A taxon of carnivorous plants Darwin never worked with is the genus *Nepenthes.* This genus contains more than 120 species; all of which possess the so-called pitchers, which are part of a metamorphosed leaf and filled with a digestive fluid. Pitcher traps provide insect prey capturing and processing, and uptake of animal-derived nutrients for most of the *Nepenthes* species. However, very few out of the many species of carnivorous *Nepenthes* plants further developed alternative strategies based on

interactions with mammals. In a mutualistic relationship between *Nepenthes lowii* and a mountain shrew, *Tupaia montana*, the plants benefit from nitrogen in the animal's feces[9]. A similar situation has been described for *Nepenthes hemsleyana* where roosting bats, *Kerivoula hardwickii*, defecate into the pitcher[10, 11]. Thus, the visiting activity of mutualistic mammalian partners significantly increased foliar nitrogen content in the host plants, *N. hemsleyana* and *N. lowii*[9, 10, 11]. However, the precise origin of the nitrogen and the biochemical background these plants use to harvest the external nitrogen trapped inside the pitchers remains unclear, so far.

Here we demonstrate that *Nepenthes* pitchers are able to absorb and use the main component of bat excrement, urea. A urease is involved as a key enzyme in urea degradation thereby releasing nitrogen in the form of ammonia. From two *Nepenthes* species the respective urease gene was cloned and the one from *N. hemsleyana* was functionally expressed. Ureases from *Nepenthes* and five other carnivorous plants from different genera are phylogenetically compared to ureases from non-carnivorous plants and fungi. The results suggest that for carnivorous plants it was not necessary to develop a specific urease for establishing a coprophagous lifestyle in *Nepenthes* but to recruit a reliable and available enzyme into an adapted nutritional context.

Results

Urea Uptake and Metabolism in *Nepenthes*

Nitrogen-containing urea is a metabolic waste product of terrestrial vertebrate animals and removed from the body in form of urine[12]. More than 70% of bats' urine dry weight consists of urea[13]. Thus, in the mutualism between bats and *Nepenthes*, the pitcher plant is provided with high amounts of urea[10, 11]. The urea-derived nitrogen is suggested to enter nitrogen metabolism in *Nepenthes* to drive growth and development[11]. To trace urea uptake and metabolism, ^{15}N-enriched urea was fed to pitchers of *N. alata*, a close but carnivorous relative of *N. hemsleyana*, and the distribution of ^{15}N was analyzed by isotope ratio mass spectrometry (IRMS). Upon ^{15}N-enriched urea application directly into the pitcher fluid, a slow time dependent increase of ^{15}N was measured in the leaf-base of the treated pitcher starting after 96 h (Fig. 1A).

An increase of ^{15}N was detectable for old as well as for young leaves sitting at the same branch of the plant (Fig. 1B). The fact that developing leaves received more ^{15}N nitrogen compared to older leaves suggests that nitrogen is preferentially directed towards developing sinks[14] (Fig. 1B). These data document that *Nepenthes* is able to take up urea from the pitcher and to allocate this feces-derived nitrogen to non-pitcher tissues[9, 10]. In plants, nitrogen released from urea hydrolysis into ammonia and CO_2 is subsequently incorporated into macromolecules. In line with this assumption, protein extracts from leaves in the same branch as the ^{15}N-enriched urea-fed pitcher exhibited higher $^{15}N/^{14}N$ ratios (Fig. 1C).

Cloning, Phylogeny and Expression of Urease from *Nepenthes*

Using a polyclonal jackbean urease antiserum for immunoblotting, in crude protein extracts from leaf and pitcher tissues of both *N. hemsleyana* and *N. alata* a cross-reacting protein was detected (Fig. 2A). Enzymatic urease activity, however, was not detectable in the pitcher fluid but measurable only in leaves extracts from *N. hemsleyana* (35 nmol NH_3 min^{-1} g fw^{-1}) and *N. alata* (20 nmol NH_3 min^{-1} g fw^{-1}).

Based on the sequences of plant urease genes, degenerate primers were designed and used for a PCR approach in *N. alata* and *N. hemsleyana*. The two full-length coding sequences for the urease genes from both *Nepenthes* species were cloned (each 2,514 bp long). They encode a single polypeptide chain with the basic characteristics of known plant ureases such as an active site that includes a nickel binding site and a jaburetox domain[15] (Fig. 2B). Both deduced protein sequences consist of 837 amino acids (predicted molecular mass of the proteins: *N. hemslyana* 90.0 kDa and *N. alata* 89.6 kDa). Sequence comparison of *Nepenthes* ureases with those from other sources indicates highly conserved amino acid residues in their catalytic sites (Supplementary Fig 1). When compared with the well-studied and crystallized *Canavalia ensiformis* urease (jackbean urease, JBU)[16], the catalytic-site residues in *Nepenthes* ureases are as follows (*Nepenthes/Canavalia*): H404/407, H406/409, K487/490, H489/492, D491/494, H516/519, H542/545, C589/592, H590/593, R606/609, D630/633, and A633/636. The active sites consist of a bi-nickel center coordinating two nickel ions. Typically, K487/490 can be carbamylated and acts as a bridging residue between the two nickel ions[16]. In all sequences of carnivorous

and non-carnivorous plants investigated here this Lysine is present; therefore the catalytic properties of all ureases are assumable. The amino acid residues that are involved in the architecture of the active site build part of a mobile flap, which acts as a gate for the substrate. In JBU, this mobile flap ranges from amino acids M590 to H607[16]. In *Nepenthes* ureases the corresponding region is also present and spans from M587 to E604 including an essential C589 (C592 in JBU) residue (Fig. 2B). Exactly in this region one amino acid, in *Nepenthes* D595, was found only in all seven carnivorous plants included in this study, but was absent in the two legume species studied and in *Arabidopsis*. In total, the urease sequences show seven of such convergent carnivorous plant-specific amino acid substitutions (Fig. 2B; Supplementary Fig. 1). Ten further amino acid changes were found to be unique for the two *Nepenthes* species (Fig. 2B; Supplementary Fig. 1).

The whole amino acid sequences of the carnivorous *N. alata* and the coprophagus *N. hemsleyana* ureases show more than 97% sequence identity at the amino acid level (Supplementary Table 1). In addition, the amino acid sequences of the two *Nepenthes* ureases share ≥86% similarity and ≥75% identity to ureases from other carnivorous plants and even to non-carnivorous plants such as *Glycine max* and *Canavalia ensiformis (*Supplementary Table 1*).* Within carnivorous plants only the urease of the endemic species *Genlisea aurea* is slightly different and shows less identity to the other ureases analyzed (Supplementary Table 1). If compared with the three non-carnivorous species, the urease sequence identity is by tend a bit lower as within the carnivorous plants, with the exception of *Cephalotus follicularis*.

In order to demonstrate the functionality of the cloned urease of *N. hemsleyana*, designated as *NhUrease*, it was transiently expressed in *Nicotiana benthamiana*, a system that has a weak urease background and that was successfully used before[17]. After six days, urease activity was measured in total plant crude extracts. To gain function, plant ureases depend on accessory proteins that help the insertion of nickel ions into their active site[17, 18]. As expected, in absence of the accessory proteins the *NhUrease* had no enzymatic activity. Upon co-expression of three urease accessary proteins from *A. thaliana*, UreD, UreF, and UreG, *NhUrease* gained function (Fig. 3). *A. thaliana* urease expressed in the same *Nicotiana benthamiana* system as "positive control" showed comparable results (Fig. 3).

158 Regarding the evolutionary age of ureases in plants, they are generally considered to
159 be as old as plants themselves but a putative horizontal transfer of prokaryotic origin to
160 unicellular algae is suggested[19]. To learn more about a putative functional evolution of
161 ureases in *Nepenthes* and other carnivorous plants' taxa, a comprehensive
162 phylogenetic analysis for eukaryotic (fungal and plant) ureases was conducted to
163 construct an evolutionary tree (Fig. 4). The ureases from *N. alata* and *N. hemsleyana*,
164 *Dionaea muscipula*, *Drosera spatulata*, and *Aldrovanda vesiculosa* were found closely
165 related (Supplementary Table 1; Fig. 4) forming part of a separate clade. This clade,
166 however, does not contain ureases from carnivorous *Genlisea aurea*[20] or *Cephalotus*
167 *follicularis*, which are found in separated clades (Fig. 4). For carnivorous *Utricularia*
168 *gibba* no urease gene was detectable (RefSeq NC_021449.1)[21].

169

170

171 **Discussion**

172 On the cellular level, the main function of plant ureases is related to nitrogen recycling
173 from endogenous urea. The presence of ureases in almost all plant taxa indicates the
174 vital role of this enzyme in metabolism, as part of arginine catabolism from which
175 arginase generates ornithine and urea[22]. In our study we showed that carnivorous
176 *Nepenthes* can harvest nitrogen in the form of urea "captured" in their pitchers and
177 released upon cleavage by the plant's urease. Thus, by employing urease not only for
178 nitrogen recycling, *Nepenthes* plants acquired a new source for nutrient
179 supplementation. Our studies with ^{15}N-enriched urea injected into closed pitchers
180 demonstrated that the exogenous urea is taken up from the pitcher fluid, hydrolyzed
181 and the nitrogen is distributed within the plant. Due to the fact that the fluid of closed
182 *Nepenthes* pitchers grown under the described conditions was shown to be sterile and
183 unsuitable for microbial growth in several species including *N. alata*[23], a possible
184 microbial involvement in urea hydrolysis in *Nepenthes* pitcher fluid could be excluded.
185 This is further supported by the fact that no urease was detected in the pitcher fluid but
186 in the tissue, thereby preventing alkalinization of the pitcher fluid by released ammonia.
187 Also, in contrast to other *Nepenthes* enzymes found in the pitcher fluid[24, 25, 26], for the
188 urease protein sequence no signal peptide was detectable that suggests secretion into
189 the digestive fluid. Thus, considering that the presence of urease in the pitcher fluid

190 was excluded, the involvement of a urea transporter is likely. Urea transporters are
191 present in most organisms and belong to different classes, some functioning as
192 passive channels, others as secondary active transporters. Interestingly, urea
193 transporters commonly found in animals and bacteria (UT, UreI, Yut) are absent in
194 plant and fungal genomes[27]. Plant and fungal genomes encode a different family of
195 urea transporters, called DUR3[28]. Whether or not *Nepenthes* pitcher tissue has DUR-
196 type urea transporters remains open and will be addressed in further studies.

197 Based on our results that a functional urease is present in the carnivorous *N. alata*, we
198 claim that urease supported a further transition from carnivory to coprophagy in the
199 closely related *N. hemsleyana* and probably *N. lowii*. In carnivorous plants, functional
200 diversification of genes and proteins, which act in quite different pathways, is
201 described. For example, class I chitinases are pathogenesis-related hydrolytic
202 enzymes in higher plants that are involved in defense against herbivory and pathogen
203 attack[29]. Interestingly, based on sequence homology, for carnivorous plants belonging
204 to the order Caryophyllales a functionalization of class I chitinases has been
205 demonstrated supporting the idea of carnivorous specialization. In that case, two
206 chitinase I subclasses evolved, very likely due to different substrate degradation
207 requirements in the chitin of fungi and insects. Thus, subclass Ia is still related to
208 pathogen defense and sub-class Ib is related to carnivory[30]. The same chitinase
209 subclass Ib has also been found recently in *Drosera*[31]. Even more striking are certain
210 ribonucleases in carnivorous plants. While S-like RNases are usually induced by
211 stress such as low nutrition level, feeding damage or even senescence, in carnivorous
212 plants their S-like ribonucleases evolved to function in carnivory[32]. Surprisingly, with
213 respect to the conventional phylogeny, the S-like ribonucleases from phylogenetically
214 non-related carnivorous plants such as *C. follicularis* on one side and *Nepenthes*,
215 *Dionaea* and *Drosera* on the other side, show higher similarities than expected. This
216 phenomenon was explained based on a carnivory-dependent evolution of the
217 enzyme[33]. Concerning the urease in *Nepenthes*, we did not find evidence of such
218 specialization at the sequence level although convergent amino acid changes for the
219 carnivorous plants could be detected (Fig. 2B), comparable with results found for S-
220 like ribonucleases[33], chitinases and phosphatases[4]. The phylogenetic tree constructed
221 in our work, including seven species of carnivorous plants, is in agreement with the
222 tree encompassing ureases from all domains of life[19]. It is also in agreement with the
223 plant phylogeny, as confirmed by comparison with the Interactive Tree of Life using the

PhyloT tool from iTOL 3.0[34]. Thus, the distant positions of *G. aurea* and *C. follicularis* ureases found in the tree - compared with the "*Nepenthes* clade" – were not surprising (Fig. 4). Interestingly, no urease was found in the genome of the carnivorous plant *Utricularia gibba*. Indeed, it has been shown that plants from this genus rely on their traps to acquire phosphates and possibly sulfates[21, 35]. Considering that nitrogen is not a limiting nutrient for this group of carnivorous plants, and that urease is not essential for nitrogen metabolism, the absence of this enzyme in such a heavily reduced genome as found for *U. gibba*[21] is comprehensible and somewhat expected.

Our findings support the hypothesis that in carnivorous plants ureases, in contrast to other enzymes such as chitinases[30], did not further evolve towards a specialized function but carry out their inherent enzymatic activity. This was not surprising in view of the fact that the substrate urea is clearly defined, in contrast to e.g. chitins from insect and fungi. The employment of urease allows metabolizing exogenously available urea present in the pitchers of *N. hemsleyana* plants. Thus, based on the adaptation of morphological structures already used for plant carnivory and basic biochemical features, a specialized coprophagous lifestyle could be established in *Nepenthes* species. However, more studies are needed to fully understand the physiology of exogenously provided urea, its uptake from the pitcher fluid, the nitrogen distribution, and its utilization within the pitcher plant.

Methods

Plant material and treatment. *Nepenthes alata* Blanco plants were grown in growth chambers at 20-25 °C, 80-85% humidity and a 16/8 h light/dark photoperiod. *Nepenthes hemsleyana* Macfarl. was grown in the greenhouse on a mixed substrate (sphagnum/bark/leaves/moss) with an average temperature of 23-25 °C and 80-100% humidity. The photoperiod was at least 12 h of light per day. For urea feeding experiments, 75 µl of 2 M urea (representing ~ 50 mM final concentration) was injected with a sterile syringe into one closed pitcher of *N. alata*. Either non-modified (^{14}N) urea (Merck) or 10% ^{15}N-enriched urea (Aldrich) was used. ^{15}N and ^{14}N content in leaf tissue were analyzed after different time points by isotope-ratio mass spectroscopy (see below). ^{14}N urea-fed and not fed plants were used as controls.

RNA isolation and cDNA synthesis. High quality total RNA was isolated from young *Nepenthes* pitcher tissue using Invitrap spin plant RNA Mini Kit (STRATEC) according to the manufacturer´s instruction. First-strand cDNA synthesis was performed using Superscript III reverse Transcriptase (Invitrogen) by using Oligo (dT)12-18 following the manufacturer`s protocol.

Cloning of *Nepenthes* urease. Eight different plant urease genes (BAB78715.1, CAC43859.1, AAO85884.1, AAA83831.1, CAC43845.1, CAC43860.1, AAN08919.1, NP_176922.1) were aligned with MEGA (v5.5) using MUSCLE algorithm[36]. Based on the alignment degenerate primers (forward: AARAATGTNHTNCCBTCWTCAAC and revers: AGGWGTDGGDATRCTNSCATTT) were designed. PCR was performed using the designed degenerate primer. A fragment of around 500 nt was amplified using cDNA from *Nepenthes* as a template. Based on that sequence we designed specific primers to obtain the full-length sequence of *Nepenthes* urease by performing RACE-PCR. *5´-RACE*: First round of 5´-RACE was performed using gene specific primer1 (GSP1: TCAGAGTCAAGTGGCCCTCTCTGCACTT) and nested gene specific primer 1 (NGSP1: GCGACCCATAGCCTGTGAATCAGAAGAGA). A second round of 5´-RACE was needed since 5´-end was incomplete after first round 5´-RACE. Based on the sequence of the first round 5´-RACE product, gene specific primer 2 (GSP2: AGACAGGCAGCTGGCGGGTACCCAGA) was designed and 5´-end of *Nepenthes* urease was amplified. *3´-RACE*: 3´-RACE gene specific primer (3´GSP: TACGAGCCGAAACCATTGCTGCAGAAGACA) was designed based on the sequence of the first round 5´-RACE product. SMART RACE cDNA amplification kit (Clontech) was used. Template RNA for RACE-PCR was isolated as described above. All PCR products were cloned into pJET1.2/blunt plasmid and sequenced. A cDNA contig was formed with seqMan and open reading frame (ORF) was determined. The complete ORF of both *N. hemsleyana* and *N. alata* ureases were amplified and cloned into pJET1.2/blunt for sequencing. Proofed sequences were submitted to EMBL.

Protein extraction, western blotting, heterologous expression, and urease activity detection. Proteins were extracted from *Nepenthes* leaf or pitcher tissue using 50 mM phosphate buffer (pH 7.5) containing 2% PVPP, 50 mM NaCl, 1 mM EDTA and 20 mM DTT (DTT was added fresh before extraction). Protein separation by SDS-PAGE and blotting were performed using Miniprotein TGX gels (Bio-Rad) and Trans blot turbo blotting system (Bio-Rad) respectively. Polyclonal anti-jackbean and

anti-*A. thaliana* urease antibodies were used for immunoblot urease detection and indicated in the particular experiments. Transient expression of *NhUrease* in *Nicotiana benthamiana* was performed according to[17]. For functional tests, *NhUrease* and accessary proteins from *A. thaliana*, UreD, UreF, and UreG, were coexpressed. Urease activity was measured as described[37].

Isotope ratio mass spectrometry analysis (IRMS). About 2 mg of dried and ground plant material was weighed with an ultra-micro balance (UMX2, Mettler-Toledo), in small 40 µl tin capsules (3.5 x 5 mm, HEKATech. HE 24005300). The capsules were sealed and combusted (oxidation at 1020 °C, reduction at 650 °C) in a constant helium stream (80 ml min^{-1}) quantitatively to CO_2, N_2 and H_2O using an elemental analyzer (EuroEA CN_2 dual, HEKAtech). After passing a water trap ($MgClO_4$) the gases were separated chromatographically at 85 °C and transferred via an open split to a coupled isotope ratio mass spectrometer (IsoPrime, Micromass). Isotope ratios were calculated as:

$$\delta^{15}N = \left[\frac{(R_{sample} - R_{standard}}{R_{standard}}\right] - 1$$

δ values usually are small numbers. Hence, they are commonly multiplied by 1000 and communicated in ‰ units or mUr[38]. R is the ratio of heavy to light isotope ($^{15}N/^{14}N$) of the sample and the standard, respectively. $\delta^{15}N$ is the relative deviation of the heavy to light isotope ratio from the international standard (air-N_2 for nitrogen). Samples were measured against our laboratory working standard alice-1 (acetanilide, $\delta^{15}N$ = -1.44 ± 0.12‰) which has been calibrated for $\delta^{15}N$ by a two-point normalization using IAEA reference material IAEA-N1 (+0.43‰) and IAEA-N_2 (+20.40‰)[39]. Empty tin capsules were used as blanks. Three technical replicates of each plant material bulk sample were analyzed. A caffeine standard ($\delta^{15}N$ = -4.01 ± 0.10‰) was analyzed together with the samples as QA reference material for long-term performance monitoring of the whole analytical setup; for details see[40]. $\delta^{15}N$ values were not corrected for *m/z* = 30 ($^{15}N_2$) because in all samples the ^{15}N content was always below 2%. For isotope ratios of leaf proteins about 2 mg of acetone-precipitated protein were used.

Phylogenetic analysis. Amino acid sequences of plant and fungal ureases were retrieved from the National Center for Biotechnology Information[41] based on a previous urease phylogeny study by Ligabue-Braun and colleagues[19]. These sequences, along

with the sequences of ureases from additional carnivorous plants presented here were aligned using MAFTT5[42] and filtered for unreliable positions using Guidance2[43]. The final alignment was used to infer the evolutionary history of these ureases by using the Maximum Likelihood method, based on the LeGascuel2008 model. A discrete Gamma distribution was used to model evolutionary rate differences among sites, and the rate variation model allowed for some sites to be evolutionarily invariable. All positions containing gaps and missing data were eliminated. Significance was assessed via 1,000 bootstrap pseudoreplicates, and branchings under 50% of bootstrap support were collapsed. All evolutionary analyses were conducted in MEGA7[44]. Sequence similarity matrices were generated with MatGAT[45].

Data availability. The assembled amino acid sequences used for the phylogenetic analysis are available from: *N. alata*, Accession #: LT622248, EMBL; *Nepenthes hemsleyana*, Acc #: LT622249, EMBL; *Dionaea muscipula*, Acc #: comp223007_c0_seq3, http://tbro.carnivorom.com[3]; *Aldrovanda vesiculosa*, Acc #: KY293301, NCBI; *Genlisea aurea*, Acc #: EPS69592, NCBI; *Drosera spatulata*, Acc #: LC194217, NCBI; *Cephalotus follicularis*, Acc #: BDDD01005981 (gene region: 27762 to 34049)[4]. The data that support the findings of this study are also available from the corresponding author on request.

338 **Acknowledgements**

339 We thank Birgit Arnold and the whole greenhouse team at the MPI for Chemical
340 Ecology as well as Ingrid Handt, Christian Ehrke and the team of the Botanical Garden
341 in Greifswald for growing *N. alata and N. hemsleyana* plants, respectively; we also
342 thank Yannick Pauchet for helpful advice and Gergő Pálfalvi, Kenji Fukushima, and
343 Yukiko Kabeya for *D. spatulata* and *C. follicularis* urease gene sequences. We thank
344 Wilhelm Boland and the Max Planck Society for continuous support. This work was
345 further supported in part by the German Academic Exchange Service (DAAD; PPP
346 Project ID 57142556) (AM), by the Brazilian agency Coordenadoria de
347 Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Program PROBRAL
348 407/2016, grant 23038.006810/2014-51 (CRC), by the German Research Foundation
349 (DFG: KE 746/5-1) (KE) and by the MEXT/JSPS KAKENHI Grant 22128001 (MH) and
350 22128002 (MH).

351

352 **Author contributions**

353 A.Y., C.R.C. and A.M. conceived and designed the research; A.Y., A.Z., S.B., and
354 C.R.S., M.G.S, and C.-P.W. performed the experiments; R.L.-B. carried out
355 phylogenetic analysis; R.H. and M.H. provided sequence information; C.R.S., M.G.S,
356 G.K. established, grew and provided *N. hemsleyana* plant material; A.Y., A.M., and
357 R.H. wrote the manuscript. All authors contributed to the manuscript.

358

359 **Competing interests**

360 The authors declare no competing financial interests.

361

362

363

References

1. Juniper BE, Robins RJ, Joel DM. *The carnivorous plants* (1989).

2. Darwin C. *Insectivorous plants* (1875).

3. Bemm F, *et al.* Venus flytrap carnivorous lifestyle builds on herbivore defense strategies. *Genome Res* **26**, 812-825 (2016).

4. Fukushima K, *et al.* The pitcher plant *Cephalotus* genome reveals genetic changes associated with carnivory. *Nat Ecol Evol* **1**, 0059 (2017).

5. Hatano N, Hamada T. Proteome analysis of pitcher fluid of the carnivorous plant Nepenthes alata. *J Proteome Res* **7**, 809-816 (2008).

6. Krausko M, *et al.* The role of electrical and jasmonate signalling in the recognition of captured prey in the carnivorous sundew plant Drosera capensis. *New Phytol*, (2016).

7. Nakamura Y, Reichelt M, Mayer VE, Mithöfer A. Jasmonates trigger prey-induced formation of 'outer stomach' in carnivorous sundew plants. *Proc R Soc Lond B Biol Sci* **280**, (2013).

8. Yilamujiang A, Reichelt M, Mithöfer A. Slow food: insect prey and chitin induce phytohormone accumulation and gene expression in carnivorous *Nepenthes* plants. *Ann Bot* **118**, 369-375 (2016).

9. Clarke CM, *et al.* Tree shrew lavatories: a novel nitrogen sequestration strategy in a tropical pitcher plant. *Biol Lett* **5**, 632-635 (2009).

10. Grafe TU, Schöner CR, Kerth G, Junaidi A, Schöner MG. A novel resource–service mutualism between bats and pitcher plants. *Biol Lett* **7**, 436-439 (2011).

11. Schöner CR, *et al.* Ecological outsourcing: a pitcher plant benefits from transferring pre-digestion of prey to a bat mutualist. *J Ecol*, (DOI: 10.1111/1365-2745.12653) (2016).

12. Wright PA. Nitrogen excretion: three end products, many physiological roles. *J Exp Biol* **198**, 273-281 (1995).

13. Hales J. Bats in churches: objective assessment of associated damage mechanisms. *Archaeol Int* **17**, 94–108 (2014).

14. Schulze W, Schulze ED, Pate JS, Gillison AN. The Nitrogen Supply from Soils and Insects during Growth of the Pitcher Plants Nepenthes mirabilis, Cephalotus follicularis and Darlingtonia californica. *Oecologia* **112**, 464-471 (1997).

15. Galvani GL, *et al.* Effect of the urease-derived peptide Jaburetox on the central nervous system of *Triatoma infestans* (Insecta: Heteroptera). *Biochim Biophys Acta* **1850**, 255-262 (2015).

16. Balasubramanian A, Ponnuraj K. Crystal structure of the first plant urease from jack bean: 83 years of journey from its first crystal to molecular structure. *J Mol Biol* **400**, 274-283 (2010).

17. Cao FQ, *et al.* Identification and characterization of proteins involved in rice urea and arginine catabolism. *Plant Physiol* **154**, 98-108 (2010).

18. Witte CP, Rosso MG, Romeis T. Identification of three urease accessory proteins that are required for urease activation in Arabidopsis. *Plant Physiol* **139**, 1155-1162 (2005).

19. Ligabue-Braun R, Andreis FC, Verli H, Carlini CR. 3-to-1: unraveling structural transitions in ureases. *Naturwissenschaften* **100**, 459-467 (2013).

20. Leushkin EV, *et al.* The miniature genome of a carnivorous plant *Genlisea aurea* contains a low number of genes and short non-coding sequences. *BMC Genom* **14**, 476 (2013).

21. Ibarra-Laclette E, *et al.* Architecture and evolution of a minute plant genome. *Nature* **498**, 94-98 (2013).

22. Witte C-P. Urea metabolism in plants. *Plant Sci* **180**, 431-438 (2011).

23. Buch F, *et al.* Secreted pitfall-trap fluid of carnivorous *Nepenthes* plants is unsuitable for microbial growth. *Ann Bot* **111**, 375-383 (2013).

24. Buch F, Kaman WE, Bikker FJ, Yilamujiang A, Mithöfer A. Nepenthesin Protease Activity Indicates Digestive Fluid Dynamics in Carnivorous *Nepenthes* Plants. *PloS One* **10**, (2015).

25. Hatano N, Hamada T. Proteomic analysis of secreted protein induced by a component of prey in pitcher fluid of the carnivorous plant *Nepenthes alata*. *J Proteomics* **75**, 4844-4852 (2012).

26. Rottloff S, *et al.* Functional characterization of a class III acid endochitinase from the traps of the carnivorous pitcher plant genus, Nepenthes. *J Exp Bot* **62**, 4639-4647 (2011).

27. Wang W-H, Köhler B, Cao F-Q, Liu L-H. Molecular and physiological aspects of urea transport in higher plants. *Plant Sci* **175**, 467-477 (2008).

28. De Michele R, Loqué D, Lalonde S, Frommer WB. Ammonium and Urea Transporter Inventory of the Selaginella and Physcomitrella Genomes. *Front Plant Sci* **3**, 62 (2012).

29. van Loon LC, Rep M, Pieterse CM. Significance of inducible defense-related proteins in infected plants. *Annu Rev Phytopathol* **44**, 135-162 (2006).

30. Renner T, Specht CD. Molecular and functional evolution of class I chitinases for plant carnivory in the caryophyllales. *Mol Biol Evol* **29**, 2971-2985 (2012).

31. Jopcik M, *et al.* Structural and functional characterisation of a class I endochitinase of the carnivorous sundew (Drosera rotundifolia L.). *Planta*, (2016).

32. Nishimura E, *et al.* S-like ribonuclease gene expression in carnivorous plants. *Planta* **238**, 955-967 (2013).

33. Nishimura E, *et al.* Structural and functional characteristics of S-like ribonucleases from carnivorous plants. *Planta* **240**, 147-159 (2014).

34. Letunic I, Bork P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res*, (2016).

35. Kibriya S, Iwan Jones J. Nutrient availability and the carnivorous habit in *Utricularia vulgaris*. *Freshwat Biol* **52**, 500-509 (2007).

36. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792-1797 (2004).

37. Witte CP, Medina-Escobar N. In-gel detection of urease with nitroblue tetrazolium and quantification of the enzyme from different crop plants using the indophenol reaction. *Anal Biochem* **290**, 102-107 (2001).

38. Brand WA, Coplen TB. Stable isotope deltas: tiny, yet robust signatures in nature. *Isotopes Environ Health Stud* **48**, 393-409 (2012).

39. Böhlke JK, Coplen TB. Interlaboratory comparison of reference materials for nitrogen-isotope-ratio measurements. In: *IAEA-TECDOC-825* (ed^(eds). International Atomic Energy Agency (1995).

40. Werner RA, Brand WA. Referencing strategies and techniques in stable isotope ratio analysis. *Rapid Commun Mass Spectrom* **15**, 501-519 (2001).

41. Agarwala R, *et al.* Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res* **44**, D7-19 (2016).

42. Katoh K, Kuma K, Miyata T, Toh H. Improvement in the accuracy of multiple sequence alignment program MAFFT. *Genome Inform* **16**, 22-33 (2005).

43. Sela I, Ashkenazy H, Katoh K, Pupko T. GUIDANCE2: accurate detection of unreliable alignment regions accounting for the uncertainty of multiple parameters. *Nucleic Acids Res* **43**, W7-14 (2015).

44. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol* **33**, 1870-1874 (2016).

45. Campanella JJ, Bitincka L, Smalley J. MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences. *BMC Bioinformatics* **4**, 29 (2003).

Legends:

Figure 1. Nitrogen uptake, mobilization and incorporation in *Nepenthes.* (A) ^{15}N uptake in leaf-base of ^{15}N-enriched urea-fed pitcher. ^{15}N-enriched urea was injected into the closed *Nepenthes alata* pitcher and ^{15}N content in the leaf base of injected pitchers was analyzed after 0, 6, 12, 24, 48, 96, 168 and 504 hours by isotope-ratio mass spectroscopy (IRMS). (B) Nitrogen mobilization from pitcher to the different leafs in the same branch. ^{15}N-enriched urea was injected into the closed *N. alata* pitcher followed by the analysis of ^{15}N content in younger and in older leaves of the same branch after 3 weeks by IRMS. Leaves 1 and 2 represent individual leaves in independent experiments. (C) ^{15}N incorporation into protein in *Nepenthes*. ^{15}N-enriched urea was injected into the closed *N. alata* pitcher. ^{15}N content in the protein extracts of young and old leaves of the same branch was analyzed after 3 weeks by IRMS. ^{14}N-fed and not fed plants were used as a control.

Figure 2. Features of urease from *Nepenthes*. (A) Assessment of urease protein in crude protein extracts from *N. hemsleyana* and *N. alata* by immunoblot using polyclonal anti-jackbean urease antibodies. (B) Schematic illustration of *Nepenthes* urease based on its amino acid (aa) sequence analysis. Active-site mobile flap and jaburetox region of *Nepenthes* urease are indicated in different colors. Positions of amino acids found to be unique for carnivorous plants' ureases when compared with *Canavalia ensiformis, Glycine max,* and *Arabidopsis thaliana* are indicated by arrows with red label; amino acids found to be unique only for *Nepenthes* ureases are indicated by arrows with black label. In this case amino acids

525 are indicated only when all non-*Nepenthes* species show an identical amino acid in that
526 position.

527

528 **Figure 3. Expression and enzymatic activity of heterologous ureases in *Nicotiana***
529 ***benthamiana*.** Proteins were transiently expressed in *Nicotiana benthamiana* for six days,
530 afterwards total proteins of the plants were extracted and desalted. (A) Specific urease activity
531 of: non-transformed *Nicotiana benthamiana*, P19, negative control; *A. thaliana* urease alone, *N.*
532 *hemsleyana* urease alone, *A. thaliana* urease with accessory proteins UreD, UreF and UreG,
533 *N. hemsleyana* urease with accessory proteins UreD, UreF and UreG. Error bars are SE (n=3)
534 (Different letters label groups which are significantly different ($p < 0.05$; Oneway Anova with
535 Turkey's post-hoc test). (B) Assessment of urease proteins in the corresponding samples by
536 immunoblot employing anti- *A. thaliana* urease-specific antiserum.

537

538 **Figure 4. Phylogenetic analysis of ureases.** Molecular phylogenetic analysis of plant (green)
539 and fungal (red) ureases, with diamonds highlighting ureases from carnivorous plants included
540 in this study. The tree with the highest log likelihood is shown. Branchings under 50% of
541 bootstrap support (1,000 pseudoreplicates) were collapsed.

542

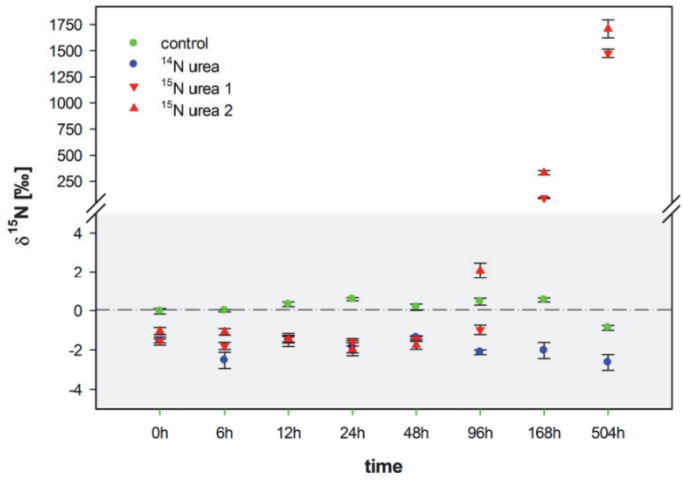
543

544 **Supplementary Fig. 1. Homology among amino acid sequences of ureases.** Included are
545 ureases from seven carnivorous plants (*Aldrovanda vesiculosa, Cephalotus follicularis,*
546 *Dionaea muscipula, Drosera spatulata, Genlisea aurea, Nepenthes alata, Nepenthes*
547 *hemsleyana*) and three non-carnivorous plants (*Canavalia ensiformis, Glycine max* embryo-
548 specific, *Arabidopsis thaliana*). Different colors indicate individual amino acids that are different
549 to the consensus amino acid at a particular position.

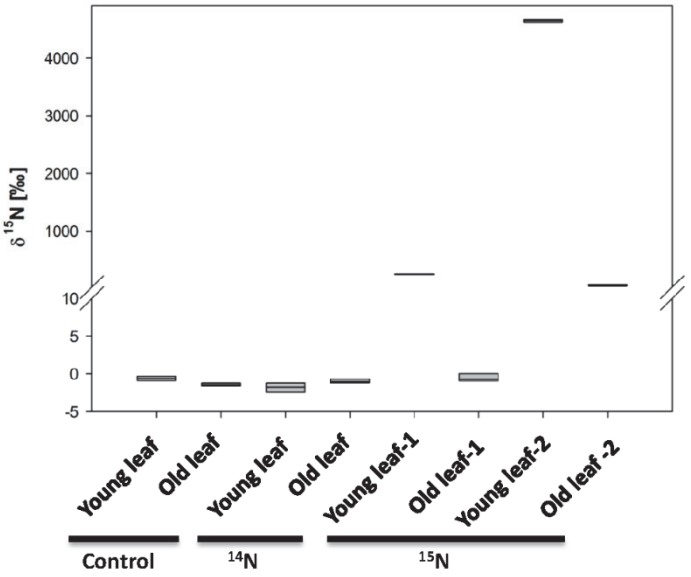
550

551

A


control
14N urea
15N urea 1
15N urea 2
δ 15N [‰]
1750
1500
1250
1000
750
500
250
4
2
0
-2
-4
0h
6h
12h
24h
48h
96h
168h
504h
time

B


δ 15N [‰]
4000
3000
2000
1000
10
5
0
-5
Young leaf
Old leaf
Young leaf
Old leaf
Young leaf-1
Old leaf-1
Young leaf-2
Old leaf -2
Control
14N
15N

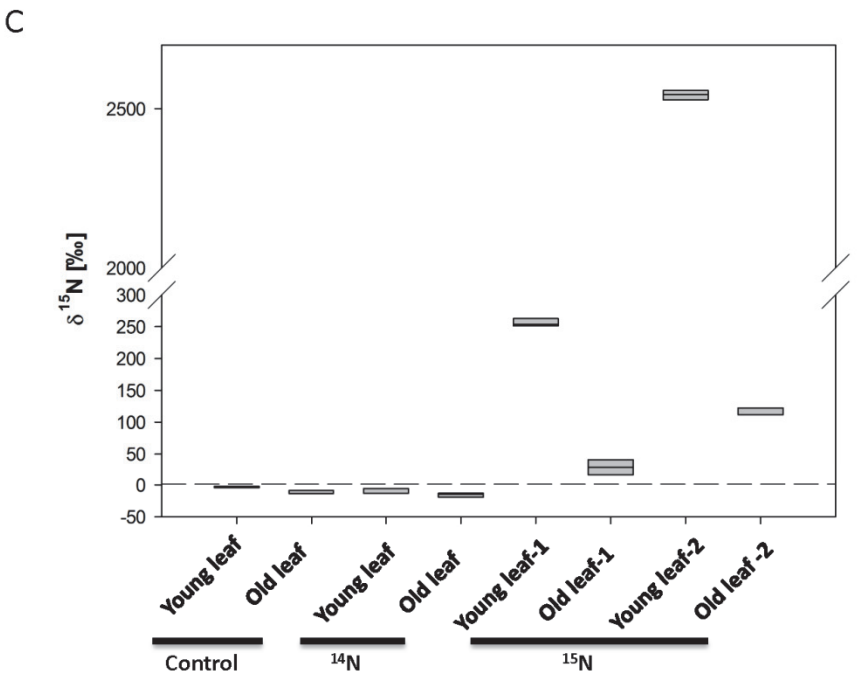


Figure 1. Nitrogen uptake, mobilization and incorporation in *Nepenthes*
(A)^{15}N uptake in leaf-base of ^{15}N labeled urea fed pitcher. ^{15}N labeled urea was injected into the closed *Nepenthes alata* pitcher and ^{15}N content in the leaf base of injected pitcher was analyzed after 0, 6, 12, 24, 48, 96, 168 and 504 hours by IRMS. (B) Nitrogen mobilization from pitcher to the different leafs in the same branch.^{15}N labeled urea was injected into the closed *Nepenthes alata* pitcher. ^{15}N content in young and old leaves of the same branch was analyzed after 3 weeks by IRMS. (C) ^{15}N incorporation into protein in *Nepenthes.*^{15}N labeled urea was injected into the closed *Nepenthes alata* pitcher. ^{15}N content in the protein extracts of young and old leaves of the same branch was analyzed after 3 weeks by IRMS. ^{14}N feed and not fed plants were used as a control.

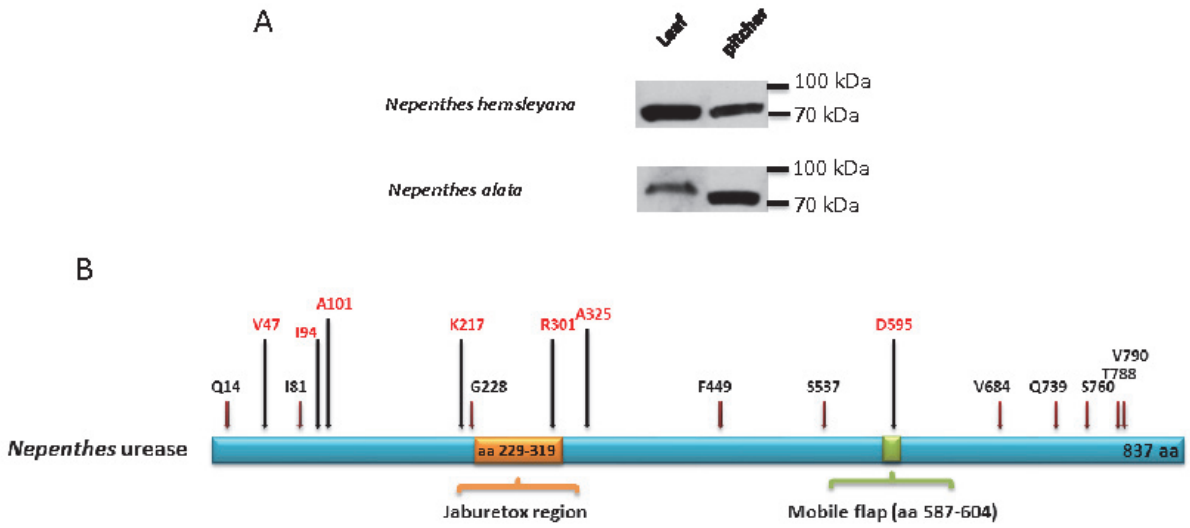


Figure 2. Features of urease from *Nepenthes*. (A) Assessment of urease protein in crude protein extracts from *N. hemsleyana* and *N. alata* by immunoblot using polyclonal anti-jackbean urease antibodies. (B) Schematic illustration of *Nepenthes* urease based on its amino acid (aa) sequence analysis. Active-site mobile flap and jaburetox region of *Nepenthes* urease are indicated in different colors. Positions of amino acids found to be unique for carnivorous plants' ureases when compared with *Canavalia ensiformis, Glycine max,* and *Arabidopsis thaliana* are indicated by arrows with red label; amino acids found to be unique only for *Nepenthes* ureases are indicated by arrows with black label. In this case amino acids are indicated only when all non-*Nepenthes* species show an identical amino acid in that position.

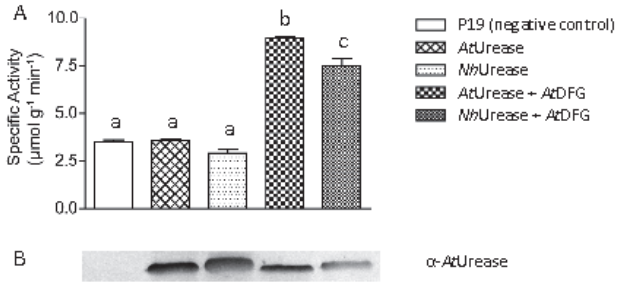


Figure 3: Enzymatic activity and expression of Ureases. Proteins were transiently expressed in *Nicotiana benthamiana* for six days, afterwards total proteins of the plants were extracted and desalted. (A) Specific urease activity of: non-transformed *Nicotiana benthamiana*, P19, negative control; *A. thaliana* urease alone, *N. hemsleyana* urease alone, *A. thaliana* urease with accessory proteins UreD, UreF and UreG, *N. hemsleyana* urease with accessory proteins UreD, UreF and UreG. Error bars are SE (n=3) (Different letters label groups which are significantly different ($p < 0.05$; Oneway Anova with Turkey's post-hoc test). (B) Assessment of urease proteins in the corresponding samples by immunoblot employing anti-*A. thaliana* urease-specific antiserum.

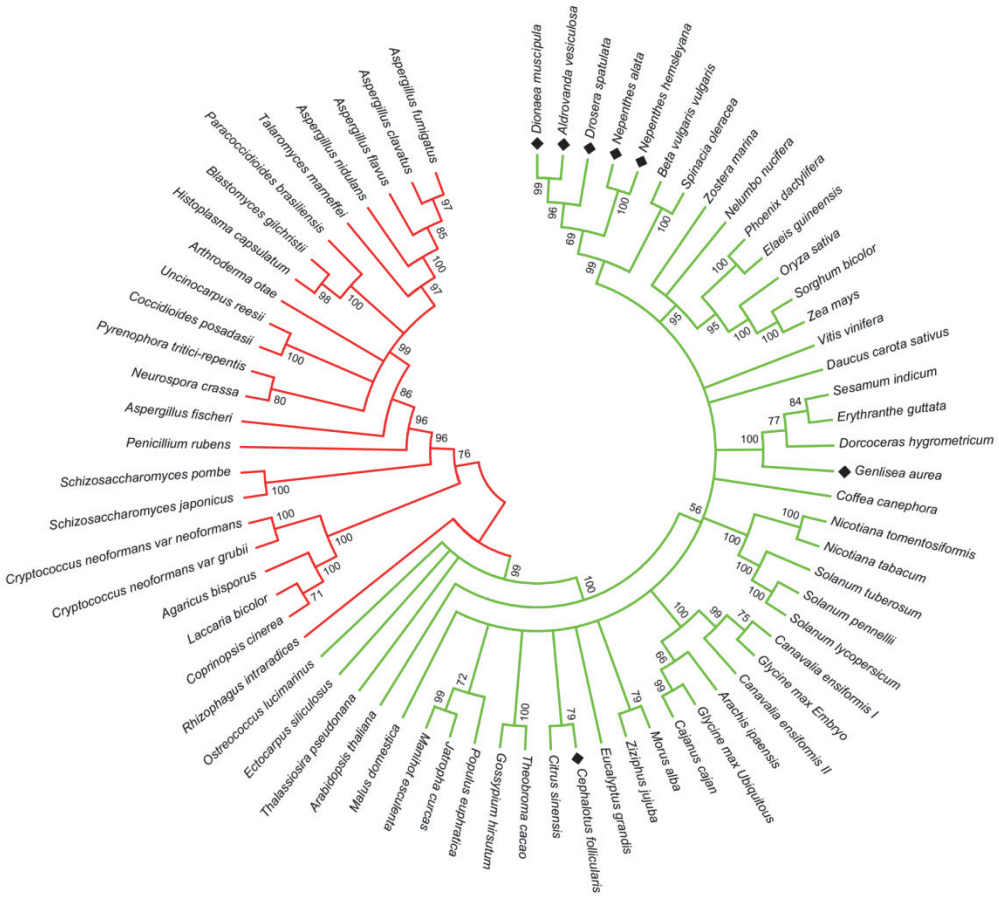


Figure 4: Molecular phylogenetic analysis of plant (green) and fungal (red) ureases, with diamonds highlighting ureases from carnivorous plants. The tree with the highest log likelihood is shown. Branchings under 50% of bootstrap support (1,000 pseudoreplicates) were collapsed.


Aldrovanda vesiculosa
Cephalotus follicularis
Dionaea muscipula
Drosera spatulata
Genlisea aurea
Nepenthes alata
Nepenthes hemsleyana
Canavalia ensiformis l
Glycine max Embryo
Arabidopsis thaliana

4. Unpublished results

Towards promoter analysis of *Nepenthes* PR genes

Promoter regions, i.e. DNA sequences located upstream of gene coding sequences, often contain *cis*-acting regulatory elements. *Cis*-acting regulatory elements play an essential role in the regulation of plant gene expression. Regulation of PR genes is often closely related to *cis*-acting regulatory elements like W-box, GCC-box and G-box (Sudisha *et al.*, 2012). Analyses of potential promoter elements of *Nepenthes* PR genes can provide more information of PR gene regulation. This implies a possibility to discover more molecular characteristics for the regulation of carnivory. For this purpose a genome walking library was constructed using *Nepenthes* genomic DNA, which allows the amplification of promoter regions of any gene. Promoter regions of selected PR genes, nepenthesin I, chitinase 3 and PR1 were investigated.

Results

Around 1 kb promoter regions were amplified for all three investigated *Nepenthes alata* PR genes, nepenthesin 1, chitinase 3 and PR1 (Figures S1 and S2). A number of WRKY binding sites are predicted on promoter regions of all three genes, including further binding sites for transcription factors like Myb and bZIB, bHLH and Dof. On the promoter regions of nepenthesin I and chitinase 3, 11 binding sites for WRKY transcription factors were predicted

for each gene. For PR 1, 27 WRKY binding sites were predicted. Around 30 Myb binding sites are present for nepenthesin I, around 20 for chitinase 3 and PR1. About 15 bZIB binding sites are predicted for chitinase 3 and PR1, but only around 5 for nepenthesin 1. The bHLH binding sites are 10 for both nepenthesin 1 and PR1, but 20 for chitinase 3. For nepenthesin I and for PR1 around 20 Dof binding sites are predicted whereas only 3 are present on the promoter region of chitinase 3.

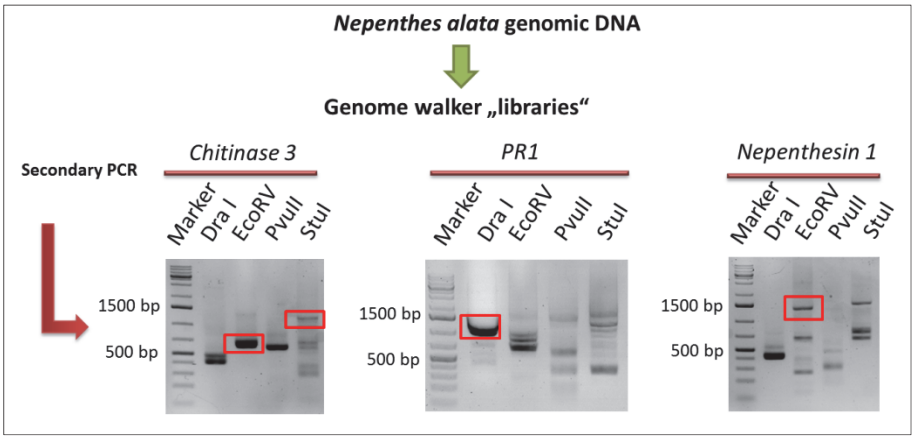
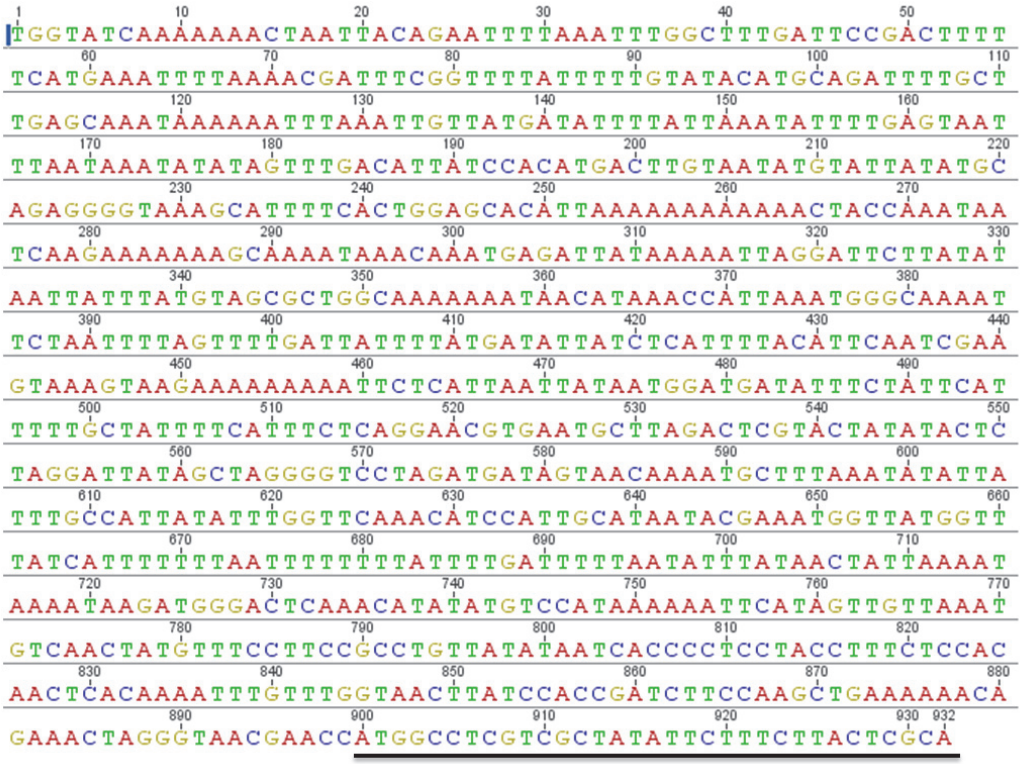


Figure S1: Schematic illustration of promoter region amplification of selected PR genes, nepenthesin I, chitinase 3 and PR1 using Genome Walker library. Each line represents amplicons from libraries which were constructed using individual restriction enzymes, *DraI*, *EcoRV*, *PvuII* and *StuI*. Red boxes indicate the amplicons used for sequencing.

A



B

C

Figure S2. Promoter regions of selected PR genes, nepenthesin I (A), chitinase 3 (B) and PR 1 (C) are shown. Overlapping regions with open reading frames (ORF) of the genes are underlined with black lines.

Methods

Genomic DNA was isolated from *Nepenthes alata* pitcher tissue using Invitrap spin plant Mini Kit (STRATEC) according to the manufacturer instruction. Genome Walker library was constructed using GenomeWalker 2.0 Kit (Clontech) following the given protocol. The following gene specific (GS) and nested gene specific primers (NGS) were used: Nepenthesin (AB266803.1):

GSP: ACTCCCCCTTTCGACAGCACGTTCTAAGA

NGSP: GCTGTTCTTGACGTAGAATGTGTTGGGGCA

Chitinase 3 (AB510164.1):

GSP: AGTTTCCGGTGGCGCAGGTGTCGGATA

NGSP: GAGCCGTGGGACGGATTGATCGAGAGAAA

Pathogenesis related protein 1 (GQ337079 was used for primer design):

GSP: GCATAAGCCGCAACAGTGTTGTTCCATGTT

NGSP: CGAGGAAGTCTTGTTTGTCGTTCTGAGCAT.

Putative *cis*-acting regulatory elements were predicted using PantPAN 2.0 (Chow *et al.*, 2016).

5. General discussion

Plants are sessile organisms that are unable to escape from unfavorable environmental conditions. Instead they need to adapt to the environment, which is supported by high plasticity of plant metabolism, pathways, and morphological variations. As an adaptation to nutrient limitations and poor soils, some plants developed a carnivorous lifestyle. Within carnivorous plants, a variety of different trapping organs with distinct morphology have been developed in different orders, families, genera and species, all of which support insect-prey based nutrient acquisition. In most carnivorous species prey digestion and subsequent degradation of nutrients rely on a mixture of hydrolytic enzymes. The exact place of prey digestion depends on the morphology of the responsible trapping organ. Species of carnivorous plants of the genus *Nepenthes* perform prey digestion in the digestive fluid of so-called pitchers, which are part of a metamorphosed leaf. Hydrolytic enzymes, which can also be categorized as members of the PR protein families, perform prey digestion in those pitchers. However, in a few *Nepenthes* species coprophagous specialization allows supplementation of a high amount of nitrogen, which is derived from mutualistic mammalian partners that defecate into the pitcher.

The objectives of my research was to investigate, on the one hand, the regulation of prey-induced digestive processes involved in nutrient supplementation in *Nepenthes* and, on the other hand, the biochemical background underlying nitrogen usage in further specialized, coprophagous species. Caught prey-depending signal initiation and induction of genes encoding proteins for the pitcher fluid were analyzed in *Nepenthes alata*. The role of phytohormones, especially the involvement of jasmonates, was studied. Additionally, promoter regions of selected PR genes were investigated. For the coprophagous plant *Nepenthes hemsleyana,* the precise origin of nitrogen and the role of ureases was examined. The results obtained have been discussed in detail in each manuscript. Here, I will discuss the results in a broader context regarding the regulation and

specialization of carnivorous lifestyle in plants, as well as its relationship to plant defense strategies in general.

Regulation of prey digestion by phytohormones

Insects have been recognized as the most significant herbivores on earth considering the fact that most of the estimated 4-6 million insect species are herbivores (Barah and Bones, 2015). Herbivory causes different kinds of damage to the plants. Mechanical damage and chemical signals are the key players for initiation of herbivory-induced plant defenses (Mithöfer and Boland, 2008). Generally, infestations of plants induce early signaling events like electrical signals followed by phytohormone biosynthesis (Gfeller *et al.*, 2010). Often jasmonates relay this information and lead to induction of gene expression. Gene expression of a particular gene can be regulated transcriptionally by a high number of transcription factors that bind to the promoter regions of a gene upon hormonal changes (Singh *et al.*, 2002). Jasmonates such as jasmonic acid, and salicylic acid are closely correlated with defense-related induction of gene expression such as genes of pathogenesis related (PR) proteins (van Loon *et al.*, 2006, Bari and Jones, 2009) that are induced as a response of various attacks (van Loon *et al.*, 2006, Sinha *et al.*, 2014).

Plants and insects have likely coexisted for the past 420 million years (Mithöfer and Boland, 2016). Supporting the assumption that the earliest appearance of carnivorous plants was at the end of Cretaceous (Juniper *et al.*, 1989) the first fossils of carnivorous plants are dated back to the Eocene, about 35-47 million years ago (Sadowski *et al.*, 2015). Thus, carnivory has definitely evolved later then plant defense against herbivory.

In carnivorous plants, the plants benefit from caught-insects as nutrient source. Hence, the carnivorous lifestyle represents a special form of plant-insect interactions. To the very heterogeneous group of carnivorous plants, there are more than 500 species known. Passive and active traps are

developed to fulfil nutrient demand; their morphology seems evolutionarily adjusted upon nutrient support, forms of nutrient supply, and ecological relevance (Juniper *et al.*, 1989).

Many aspects of plant defense pathways, especially the early events (Maffei *et al.*, 2007) in successful defense against insect herbivory seem to be present in carnivory-related processes in plants. The fruit fly *Drosophila melanogaster*, was used as an insect-prey model in the pitcher fluid of *N. alata. D. melanogaster* induced both, an elevation of jasmonate phytohormones as well as the expression of PR genes in pitcher tissue (Manuscript II). Measured by an optimized FRET (fluorescent resonance energy transfer)-based technique, proteolytic activity of prominent pitcher fluid protein, nepenthesin, was also demonstrated to be induced by prey. In addition, external JA, rather than SA application induced pitcher fluid proteolytic activity (Manuscript I). This corresponds to earlier studies where trapped prey in *Drosera* and *Dionaea* activated jasmonate signaling as well (Escalante-Perez *et al.*, 2011, Nakamura *et al.*, 2013). Moreover, both chemical and mechanical stimuli were important for JA signaling activation in *Drosera* and *Dionaea* (Mithöfer *et al.*, 2014, Böhm *et al.*, 2016, Krausko *et al.*, in press). In *Drosera*, the prey-initiated tentacle movement caused electrical signals followed by jasmonate accumulation (Krausko *et al.*, in press). Similarly in *Dionaea muscipula*, JA signaling is induced by trigger-hair-generated action potentials (APs) (Böhm *et al.*, 2016). In the case of *Nepenthes* results of mechanical stimulation (or in combination with chemical stimuli) are lacking. But exogenously applied chitin, a component of insect exoskeleton, was sufficient for jasmonate-mediated initiation of the carnivory signaling (Manuscript II). However, all these results demonstrate that carnivorous plants use the available JA signaling pathway in their trapping organs. JA-induced gene expression contributes to prey-digestion and nutrient acquisition. Participation of JA signaling in these processes strongly supports the hypothesis that plant carnivory is evolved from plant defense mechanisms (Mithöfer, 2011, Nakamura *et al.*, 2013, Mithöfer *et al.*, 2014, Bemm *et al.*, 2016, Krausko *et al.*, in press). This is also supported by the

recently published transcriptome landscape of *Dionaea* (Bemm *et al.*, 2016). However, detailed steps in signaling cascades from prey recognition to nutrient uptake are not completely understood in any carnivorous plant. For carnivorous plants the jasmonate pathway seems to be, not only a main modulator, but also an intersection coordinator, probably with some adaptations. It should be mentioned that prey-induced JA-accumulation in carnivorous plants showed a distinct time course that differs from herbivory-induced JA accumulation (Manuscript II) (Maffei *et al.*, 2007, Nakamura *et al.*, 2013). In fact, prey-induced jasmonate accumulation, gene expression and enzyme accumulation in *Nepenthes* is delayed by hours (Manuscript II) (Maffei *et al.*, 2007).

Clear evidences for involvement of jasmonates in many carnivory-modulated processes in recently investigated carnivorous plants lets us assume an even broader participation of jasmonate signaling in further nutrient acquisition steps such as endocytosis. In many carnivorous plants endocytosis plays a key role in nutrient uptake by enabling absorption and intracellular digestion of even whole proteins (Adlassnig *et al.*, 2012). Jasmonates are involved in modulation of endocytosis in plants (Sun *et al.*, 2011). Therefore it is tempting to speculate that in carnivorous plants, jasmonates are also involved in endocytosis mediated nutrient uptake. Further research is needed to prove a direct link between jasmonates and endocytosis, and molecular players of endocytosis in carnivorous plants needs to be identified.

As a response to prey or chitin in the pitcher fluid, *Nepenthes* PR genes selectively respond in pitcher tissue (Manuscript II). Under pathogen or insect attack many PR proteins are induced, which is mainly mediated by the induction of JA, SA or ethylene (van Loon *et al.*, 2006). JA- or SA-dependent selective induction of PR genes was demonstrated in tobacco leaves (Niki *et al.*, 1998). Crosstalk between JA and SA, especially in the context of plant pathogen interaction and induction of defense response upon pathogen attack is antagonistic (Robert-Seilaniantz *et al.*, 2011). This may explain the selective and precise time course coordination between gene expression and

hormonal induction during prey digestion in *Nepenthes*. Promoter analysis of PR genes in *Nepenthes,* especially the identification and characterization of *cis*-acting regulatory elements may provide more insight in PR gene regulation in *Nepenthes*, and in plant carnivory in general.

Cis-acting regulatory elements play an essential role in the regulation of plant gene expression. Combination of *cis*-acting regulatory elements and transcription factors allows an extremely specific gene expression regulation upon stress stimulation in plants (Hernandez-Garcia and Finer, 2014, Porto *et al.*, 2014, Biłas *et al.*, 2016). Regulation of PR genes is often closely related to *cis*-acting regulatory elements like W-box, GCC-box and G-box (Sudisha *et al.*, 2012). W-box, for instance, is a binding site for a class of transcription factors called WRKY(s) (Phukan *et al.*, 2016). Large number of WRKYs regulate multiple responses through various interconnecting signaling networks in plants (Phukan *et al.*, 2016).

To analyze the promotors of different PR genes in *Nepenthes*, a Genome Walker library was constructed (see unpublished results). Primarily, promoter regions of PR genes like nepenthesin I, chitinase 3 and PR 1 were investigated. Promoter regions of selected PR genes contained a number of binding sites for WRKY transcription factors and additional promoter regulatory regions like Myb and bZIB, which are known to play a role in the regulation of plant defense related gene expression (Singh *et al.*, 2002) (see unpublished results). On the promoter regions of nepenthesin I and chitinase 3, 11 binding sites for WRKY transcription factors for each gene were predicted. Furthermore, 27 WRKY binding sites were predicted for PR 1. The same classes of binding sites were reported for chitinase genes from *Drosera*, which were functionalized to act in carnivory (Jopcik *et al.*, 2016). The presence of both JA- and SA-responsive WRKY transcriptional factors was identified in other plants including *Arabidopsis* (Dong *et al.*, 2003, Schluttenhofer *et al.*, 2014). Recently it was shown that WRKY22 modulates the interplay between JA and SA signaling in *Arabidopsis* (Kloth *et al.*, 2016). Further experimental investigation is needed to prove the exact regulatory

mechanisms and functionality of all predicted *Nepenthes cis*- acting regulatory elements. A deletion analysis with truncated promoters (Oropeza-Aburto *et al.*, 2011) can be performed for further characterization.

The more we learn, and the more we design the future research topics, the more comprehensively we can understand carnivorous plants. Knowledge about protein composition of *Nepenthes* pitcher fluid and its biochemical properties deepens our understanding of the molecular features of a carnivorous lifestyle. Known protein characteristics may help to develop an ideal way to discover new proteins. Glycosylation of the heterologously expressed nepenthesin cloned from *Nepenthes* is shown (Manuscript I). High glycosylation of PR1 from the pitcher fluid of *Nepenthes* was proved (Buch *et al.*, 2014). Glycosylation sites for more known PR proteins from *Nepenthes* are also predicted. Much more proteins in the pitcher fluid are possibly glycosylated, as it may have a protective role in harsh conditions. *Nepenthes* pitcher fluid exhibits a special environment due to extreme pH change, necessary for prey digestion (Manuscript I). Further studies of glycosylation pattern of enzymes from pitcher fluids might increase the possibility to discover canonical biochemical properties of the proteins. This could open new possibilities for biotechnological applications. Using chromatographic methods applying concanavalin A (Con A) in combination with proteomic approaches may allow the identification of new protein components from the pitcher fluid, which were not yet detectible due to their low amount in the pitcher fluids.

Further specialization in carnivorous plants

Apart from the above described adaptations of insect-prey based carnivory, further specialization exists in carnivorous plants, which enables them to use distinct nutrient sources. A mutualism-based nitrogen access in *N. hemsleyana* is an example of this phenomenon. The results regarding urease-mediated urea usage support the idea that further specialization leads to coprophagy in *Nepenthes* (Manuscript III). However, whether or not these plants rely completely on feces-derived nitrogen or still have the ability

to catch and digest insect prey was not completely understood. Interestingly, *N. hemsleyana* plants fed with feces demonstrated increased survival, growth and photosynthesis compared to the arthropod fed plants (Schöner *et al.*, in press). Thus, the authors hypothesized an outsourcing strategy where the plant benefits from prey that was pre-digested by the bat mutualist (Schöner *et al.*, in press).

From the evolutionary point of view, carnivory in plants is assumed to have evolved independently nine times in five different orders of higher plants (Ellison and Gotelli, 2009, Givnish, 2015). In carnivorous plants, functional diversification of genes involved in prey digestion is observed (Renner and Specht, 2012, Nishimura *et al.*, 2014, Jopcik *et al.*, 2016). Carnivory-specific functionalization and sub-functionalization of some enzymes like chitinase and S-like ribonuclease in favor of carnivory was reported (Renner and Specht, 2012, Nishimura et al., 2014). In some carnivorous plants in the order of Caryophyllales, functionalization of class I chitinases for carnivory has been demonstrated. Two subclasses, subclass 1a and subclass 1b have evolved. Function of subclass 1a remained to act for plant defense, where subclass 1b is related to plant carnivory (Renner and Specht, 2012, Jopcik et al., 2016). S-like ribonucleases also evolved to function in carnivory, rather than function in self-incompatibility mechanism of plant, compared to its sequence homology S-RNases (Nishimura *et al.*, 2013). Carnivorous plant S-like ribonucleases exhibited specific amino acid residues unique for carnivorous plants. In the case of urease, *Nepenthes* ureases demonstrated the same functional domain characteristics like other plant ureases and seem to represent a typical urease wihout carnivory-related features. This might be due to the fact that the urease is not part of the digestive fluid and, thus, not exposed to acidic conditions and enormous proteolytic activities. However, urease supported further specialization from carnivory to coprophagy in *Nepenthes*. Phylogenetic tree constructed for urease is in agreement with the phylogenetic relationship of the included carnivorous plants. Nevertheless,

the urease sequences studied show seven carnivorous plant-specific amino acid substitutions. Ten amino acid changes were found to be unique for the two *Nepenthes* species (Manuscript III). Convergent evolution of amino acid substitution specifically for carnivory, or even for coprophagy, cannot be excluded completely, by taking those carnivorous plant specific amino acid changes in urease into account. Considering the described carnivory specific functionalization and evolution of chitinase and S-like ribonuclease, convergent evolution seems to be a driving force for development of carnivory strategies under ecological adaptations.

Interestingly, for the aquatic carnivorous plant *Utricularia gibba* no urease gene was detected (Manuscript III). Environmental factors such as nitrogen availability in aquatic ecosystems most probably played a role in complete urease gene loss in *U. gibba*. Hence, ecological adaptation by urease usage in carnivorous plants cannot be excluded.

The results of this thesis concerning the regulation of prey digestion in *Nepenthes* demonstrate versatility of jasmonate signaling pathway in prey derived nutrient supplementation. These results confirm the hypothesis that carnivory in plants has evolved from plant defense strategies, which is further strongly supported by the recent research achievements from other carnivorous plants (Mithöfer, 2011, Nakamura *et al.*, 2013, Mithöfer *et al.*, 2014, Bemm *et al.*, 2016, Krausko *et al.*, in press). It also shows how shaping of existing pathways can contribute to developing new lifestyles, here in favor of carnivory plants.

6. References

Adlassnig W, Koller-Peroutka M, Bauer S, Koshkin E, Lendl T, Lichtscheidl IK. 2012. Endocytotic uptake of nutrients in carnivorous plants. *Plant Journal,* **71**: 303-313, doi:10.1111/j.1365-313X.2012.04997.x.

An CI, Fukusaki E, Kobayashi A. 2002. Aspartic proteinases are expressed in pitchers of the carnivorous plant *Nepenthes alata* Blanco. *Planta,* **214**: 661-667, doi:10.1007/s004250100665.

Athauda SBP, Matsumoto K, Rajapakshe S, Kuribayashi M, Kojima M, Kubomura-Yoshida N, Iwamatsu A, Shibata C, Inoue H, Takahashi K. 2004. Enzymic and structural characterization of nepenthesin, a unique member of a novel subfamily of aspartic proteinases. *Biochemical Journal,* **381**: 295-306, doi:10.1042/bj20031575.

Balasubramanian A, Ponnuraj K. 2010. Crystal structure of the first plant urease from jack bean: 83 years of journey from its first crystal to molecular structure. *Journal of Molecular Biology,* **400**: 274-83, doi:10.1016/j.jmb.2010.05.009.

Barah P, Bones AM. 2015. Multidimensional approaches for studying plant defence against insects: from ecology to omics and synthetic biology. *Journal of Experimental Botany,* **66**: 479-93, doi:10.1093/jxb/eru489.

Bari R, Jones JD. 2009. Role of plant hormones in plant defence responses. *Plant Molecular Biology,* **69**: 473-88, doi:10.1007/s11103-008-9435-0.

Bemm F, Becker D, Larisch C, Kreuzer I, Escalante-Perez M, Schulze WX, Ankenbrand M, Van de Weyer AL, Krol E, Al-Rasheid KA, Mithöfer A, Weber AP, Schultz J, Hedrich R. 2016. Venus flytrap carnivorous lifestyle builds on herbivore defense strategies. *Genome Research,* **26**: 812-25, doi:10.1101/gr.202200.115.

Biłas R, Szafran K, Hnatuszko-Konka K, Kononowicz AK. 2016. Cis-regulatory elements used to control gene expression in plants. *Plant Cell, Tissue and Organ Culture (PCTOC),* **127**: 269-287, doi:10.1007/s11240-016-1057-7.

Böhm J, Scherzer S, Krol E, Kreuzer I, von Meyer K, Lorey C, Mueller TD, Shabala L, Monte I, Solano R, Al-Rasheid KA, Rennenberg H, Shabala S, Neher E, Hedrich R. 2016. The Venus Flytrap Dionaea muscipula Counts Prey-Induced Action Potentials to Induce Sodium Uptake. *Current Biology,* **26**: 286-95, doi:10.1016/j.cub.2015.11.057.

Buch F, Pauchet Y, Rott M, Mithöfer A. 2014. Characterization and heterologous expression of a PR-1 protein from traps of the carnivorous plant *Nepenthes mirabilis*. *Phytochemistry,* **100**: 43-50, doi:10.1016/j.phytochem.2014.01.014.

Chow CN, Zheng HQ, Wu NY, Chien CH, Huang HD, Lee TY, Chiang-Hsieh YF, Hou PF, Yang TY, Chang WC. 2016. PlantPAN 2.0: an update of plant promoter analysis navigator for reconstructing transcriptional regulatory networks in plants. *Nucleic Acids Research,* **44**: D1154-60, doi:10.1093/nar/gkv1035.

Clarke C, Moran JA, Chin L. 2010. Mutualism between tree shrews and pitcher plants: Perspectives and avenues for future research. *Plant Signaling & Behavior,* **5**: 1187-9, doi:10.4161/psb.5.10.12807.

Clarke CM, Bauer U, Lee CiC, Tuen AA, Rembold K, Moran JA. 2009. Tree shrew lavatories: a novel nitrogen sequestration strategy in a tropical pitcher plant. *Biology Letters,* **5**: 632-635, doi:10.1098/rsbl.2009.0311.

Darwin C. 1875. *Insectivorous plants.* London: John Murray, pp. x, 462.

Dong J, Chen C, Chen Z. 2003. Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. *Plant Molecular Biology,* **51**: 21-37, doi:10.1023/A:1020780022549.

Eilenberg H, Pnini-Cohen S, Schuster S, Movtchan A, Zilberstein A. 2006. Isolation and characterization of chitinase genes from pitchers of the carnivorous plant *Nepenthes khasiana*. *Journal of Experimental Botany,* **57**: 2775-84, doi:10.1093/jxb/erl048.

Ellison AM, Gotelli NJ. 2009. Energetics and the evolution of carnivorous plants--Darwin's 'most wonderful plants in the world'. *Journal of Experimental Botany,* **60**: 19-42, doi:10.1093/jxb/ern179.

Elser JJ, Fagan WF, Subramanian S, Kumar S. 2006. Signatures of ecological resource availability in the animal and plant proteomes. *Molecular Biology and Evolution,* **23**: 1946-1951, doi:10.1093/molbev/msl068.

Escalante-Perez M, Krol E, Stange A, Geiger D, Al-Rasheid KA, Hause B, Neher E, Hedrich R. 2011. A special pair of phytohormones controls excitability, slow closure, and external stomach formation in the Venus flytrap. *Proceedings of the National Academy of Sciences of the United States of America,* **108**: 15492-7, doi:10.1073/pnas.1112535108.

Gfeller A, Liechti R, Farmer EE. 2010. Arabidopsis jasmonate signaling pathway. *Sci Signal,* **3**: cm4, doi:10.1126/scisignal.3109cm4.

Givnish TJ. 2015. New evidence on the origin of carnivorous plants. *Proceedings of the National Academy of Sciences of the United States of America,* **112**: 10-1, doi:10.1073/pnas.1422278112.

Grafe TU, Schöner CR, Kerth G, Junaidi A, Schöner MG. 2011. A novel resource–service mutualism between bats and pitcher plants. *Biology Letters,* **7**: 436-439, doi:10.1098/rsbl.2010.1141.

Greenwood M, Clarke C, Lee CC, Gunsalam A, Clarke RH. 2011. A unique resource mutualism between the giant Bornean pitcher plant, *Nepenthes rajah*, and members of a small mammal community. *PLoS One,* **6**: e21114, doi:10.1371/journal.pone.0021114.

Hales J. 2014. Bats in churches: objective assessment of associated damage mechanisms. *Archaeology International,* **17**: 94–108, doi:10.5334/ai.1703.

Hatano N, Hamada T. 2008. Proteome analysis of pitcher fluid of the carnivorous plant *Nepenthes alata*. *Journal of Proteome Research,* **7**: 809-816, doi:10.1021/pr700566d.

Hatano N, Hamada T. 2012. Proteomic analysis of secreted protein induced by a component of prey in pitcher fluid of the carnivorous plant *Nepenthes alata*. *Journal of Proteomics,* **75**: 4844-4852, doi:10.1016/j.jprot.2012.05.048.

Hernandez-Garcia CM, Finer JJ. 2014. Identification and validation of promoters and cis-acting regulatory elements. *Plant Science,* **217–218**: 109-119, doi:10.1016/j.plantsci.2013.12.007.

Ibanez F, Wall L, Fabra A. 2016. Starting points in plant-bacteria nitrogen-fixing symbioses: intercellular invasion of the roots. *Journal of Experimental Botany*: erw387, doi:10.1093/jxb/erw387.

Jiang L, Wu J, Fan S, Li W, Dong L, Cheng Q, Xu P, Zhang S. 2015. Isolation and Characterization of a Novel Pathogenesis-Related Protein Gene (GmPRP) with Induced Expression in Soybean (Glycine max) during Infection with Phytophthora sojae. *PLoS One,* **10**: e0129932, doi:10.1371/journal.pone.0129932.

Jopcik M, Moravcikova J, Matusikova I, Bauer M, Rajninec M, Libantova J. 2016. Structural and functional characterisation of a class I endochitinase of the carnivorous sundew (Drosera rotundifolia L.). *Planta,* **245**: 313-327, doi:10.1007/s00425-016-2608-1.

Jung HW, Kim KD, Hwang BK. 2005. Identification of pathogen-responsive regions in the promoter of a pepper lipid transfer protein gene (CALTPI) and the enhanced

resistance of the CALTPI transgenic Arabidopsis against pathogen and environmental stresses. *Planta,* **221**: 361-373, doi:10.1007/s00425-004-1461-9.

Juniper BE, Robins RJ, Joel DM. 1989. *The carnivorous plants*, pp. i-xii, 1-353.

Kadek A, Mrazek H, Halada P, Rey M, Schriemer DC, Man P. 2014. Aspartic Protease Nepenthesin-1 as a Tool for Digestion in Hydrogen/Deuterium Exchange Mass Spectrometry. *Analytical Chemistry,* **86**: 4287-4294, doi:10.1021/ac404076j.

Kitajima S, Sato F. 1999. Plant pathogenesis-related proteins: molecular mechanisms of gene expression and protein function. *Journal of Biochemistry (Tokyo),* **125**: 1-8.

Kloth KJ, Wiegers GL, Busscher-Lange J, van Haarst JC, Kruijer W, Bouwmeester HJ, Dicke M, Jongsma MA. 2016. AtWRKY22 promotes susceptibility to aphids and modulates salicylic acid and jasmonic acid signalling. *Journal of Experimental Botany,* **67**: 3383-3396, doi:10.1093/jxb/erw159.

Krausko M, Perutka Z, Sebela M, Samajova O, Samaj J, Novak O, Pavlovic A. in press. The role of electrical and jasmonate signalling in the recognition of captured prey in the carnivorous sundew plant Drosera capensis. *New Phytologist*, doi:10.1111/nph.14352.

Lee L, Zhang Y, Ozar B, Sensen CW, Schriemer DC. 2016. Carnivorous Nutrition in Pitcher Plants (*Nepenthes* spp.) via an Unusual Complement of Endogenous Enzymes. *Journal of Proteome Research,* **15**: 3108-17, doi:10.1021/acs.jproteome.6b00224.

Libiakova M, Flokova K, Novak O, Slovakova L, Pavlovic A. 2014. Abundance of cysteine endopeptidase dionain in digestive fluid of Venus flytrap (*Dionaea muscipula* Ellis) is regulated by different stimuli from prey through jasmonates. *PLoS One,* **9**: e104424, doi:10.1371/journal.pone.0104424.

Maffei ME, Mithöfer A, Boland W. 2007. Before gene expression: early events in plant-insect interaction. *Trends in Plant Science,* **12**: 310-6, doi:10.1016/j.tplants.2007.06.001.

Mithöfer A. 2011. Carnivorous pitcher plants: Insights in an old topic. *Phytochemistry,* **72**: 1678-1682, doi:10.1016/j.phytochem.2010.11.024.

Mithöfer A, Boland W. 2008. Recognition of Herbivory-Associated Molecular Patterns. *Plant Physiology,* **146**: 825-831, doi:10.1104/pp.107.113118.

Mithöfer A, Boland W. 2012. Plant defense against herbivores: chemical aspects. *Annual Review of Plant Biology,* **63**: 431-50, doi:10.1146/annurev-arplant-042110-103854.

Mithöfer A, Boland W. 2016. Do you speak chemistry? Small chemical compounds represent the evolutionary oldest form of communication between organisms. *EMBO Rep,* **17**: 626-9, doi:10.15252/embr.201642301.

Mithöfer A, Reichelt M, Nakamura Y. 2014. Wound and insect-induced jasmonate accumulation in carnivorous Drosera capensis: two sides of the same coin. *Plant Biology,* **16**: 982-7, doi:10.1111/plb.12148.

Nakamura Y, Reichelt M, Mayer VE, Mithöfer A. 2013. Jasmonates trigger prey-induced formation of 'outer stomach' in carnivorous sundew plants. *Proceedings of the Royal Society B: Biological Sciences,* **280**, doi:10.1098/rspb.2013.0228.

Niki T, Mitsuhara I, Seo S, Ohtsubo N, Ohashi Y. 1998. Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant and Cell Physiology,* **39**: 500-507.

Nishimura E, Jumyo S, Arai N, Kanna K, Kume M, Nishikawa J, Tanase J, Ohyama T. 2014. Structural and functional characteristics of S-like ribonucleases from carnivorous plants. *Planta,* **240**: 147-59, doi:10.1007/s00425-014-2072-8.

Nishimura E, Kawahara M, Kodaira R, Kume M, Arai N, Nishikawa J, Ohyama T. 2013. S-like ribonuclease gene expression in carnivorous plants. *Planta,* **238**: 955-67, doi:10.1007/s00425-013-1945-6.

Oropeza-Aburto A, Cruz-Ramírez A, Acevedo-Hernández GJ, Pérez-Torres C-A, Caballero-Pérez J, Herrera-Estrella L. 2011. Functional analysis of the Arabidopsis PLDZ2 promoter reveals an evolutionarily conserved low-Pi-responsive transcriptional enhancer element. *Journal of Experimental Botany,* **63**: 2189-2202, doi:10.1093/jxb/err446.

Phukan UJ, Jeena GS, Shukla RK. 2016. WRKY Transcription Factors: Molecular Regulation and Stress Responses in Plants. *Frontiers in Plant Science,* **7**: 760, doi:10.3389/fpls.2016.00760.

Porto MS, Pinheiro MP, Batista VG, dos Santos RC, Filho Pde A, de Lima LM. 2014. Plant promoters: an approach of structure and function. *Molecular Biotechnology,* **56**: 38-49, doi:10.1007/s12033-013-9713-1.

Renner T, Specht CD. 2012. Molecular and functional evolution of class I chitinases for plant carnivory in the caryophyllales. *Molecular Biology and Evolution,* **29**: 2971-85, doi:10.1093/molbev/mss106.

Rey M, Yang M, Lee L, Zhang Y, Sheff JG, Sensen CW, Mrazek H, Halada P, Man P, McCarville JL, Verdu EF, Schriemer DC. 2016. Addressing proteolytic efficiency in enzymatic degradation therapy for celiac disease. *Scientific Reports,* **6**: 30980, doi:10.1038/srep30980.

Robert-Seilaniantz A, Grant M, Jones JD. 2011. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annual Review of Phytopathology,* **49**: 317-43, doi:10.1146/annurev-phyto-073009-114447.

Rottloff S, Miguel S, Biteau F, Nisse E, Hammann P, Kuhn L, Chicher J, Bazile V, Gaume L, Mignard B, Hehn A, Bourgaud F. 2016. Proteome analysis of digestive fluids in *Nepenthes* pitchers. *Annals of Botany,* **117**: 479-95, doi:10.1093/aob/mcw001.

Rottloff S, Stieber R, Maischak H, Turini FG, Heubl G, Mithöfer A. 2011. Functional characterization of a class III acid endochitinase from the traps of the carnivorous pitcher plant genus, *Nepenthes*. *Journal of Experimental Botany,* **62**: 4639-4647, doi:10.1093/jxb/err173.

Sadowski E-M, Seyfullah LJ, Sadowski F, Fleischmann A, Behling H, Schmidt AR. 2015. Carnivorous leaves from Baltic amber. *Proceedings of the National Academy of Sciences of the United States of America,* **112**: 190-195, doi:10.1073/pnas.1414777111.

Schluttenhofer C, Pattanaik S, Patra B, Yuan L. 2014. Analyses of Catharanthus roseus and Arabidopsis thaliana WRKY transcription factors reveal involvement in jasmonate signaling. *BMC Genomics,* **15**: 502, doi:10.1186/1471-2164-15-502.

Schöner CR, Schöner MG, Grafe TU, Clarke CM, Dombrowski L, Tan MC, Kerth G. in press. Ecological outsourcing: a pitcher plant benefits from transferring pre-digestion of prey to a bat mutualist. *Journal of Ecology*, doi:10.1111/1365-2745.12653.

Schöner MG, Schöner CR, Simon R, Grafe TU, Puechmaille SJ, Ji LL, Kerth G. 2015. Bats Are Acoustically Attracted to Mutualistic Carnivorous Plants. *Current Biology,* **25**: 1911-6, doi:10.1016/j.cub.2015.05.054.

Singh KB, Foley RC, Oñate-Sánchez L. 2002. Transcription factors in plant defense and stress responses. *Current Opinion in Plant Biology,* **5**: 430-436, doi:10.1016/S1369-5266(02)00289-3.

Sinha M, Singh RP, Kushwaha GS, Iqbal N, Singh A, Kaushik S, Kaur P, Sharma S, Singh TP. 2014. Current overview of allergens of plant pathogenesis related protein families. *ScientificWorldJournal,* **2014**: 543195, doi:10.1155/2014/543195.

Spoel SH, Dong X. 2012. How do plants achieve immunity? Defence without specialized immune cells. *Nature Reviews Immunology,* **12**: 89-100, doi:10.1038/nri3141.

Stephenson P, Hogan J. 2006. Cloning and Characterization of a Ribonuclease, a Cysteine Proteinase, and an Aspartic Proteinase from Pitchers of the Carnivorous Plant *Nepenthes ventricosa* Blanco. *International Journal of Plant Sciences,* **167**: 239-248, doi:10.1086/499284.

Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B. 1993. Plant 'pathogenesis-related' proteins and their role in defense against pathogens. *Biochimie,* **75**: 687-706, doi:10.1016/0300-9084(93)90100-7.

Sudisha J, Sharathchandra RG, Amruthesh KN, Kumar A, Shetty HS. 2012. Pathogenesis Related Proteins in Plant Defense Response. In: Mérillon JM, Ramawat KG, eds. *Plant Defence: Biological Control*. Dordrecht: Springer Netherlands, pp. 379-403.

Sun J, Chen Q, Qi L, Jiang H, Li S, Xu Y, Liu F, Zhou W, Pan J, Li X, Palme K, Li C. 2011. Jasmonate modulates endocytosis and plasma membrane accumulation of the Arabidopsis PIN2 protein. *New Phytologist,* **191**: 360-75, doi:10.1111/j.1469-8137.2011.03713.x.

Takahashi K. 2007. Variations in the content and isozymic composition of nepenthesin in the pitcher fluids among *Nepenthes* species. *Carnivorous Plant Newsletter,* **36**: 73-76.

van Loon LC, Rep M, Pieterse CM. 2006. Significance of inducible defense-related proteins in infected plants. *Annual Review of Phytopathology,* **44**: 135-62, doi:10.1146/annurev.phyto.44.070505.143425.

van Loon LC, van Strien EA. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology,* **55**: 85-97, doi:10.1006/pmpp.1999.0213.

Verma V, Ravindran P, Kumar PP. 2016. Plant hormone-mediated regulation of stress responses. *BMC Plant Biology,* **16**: 86, doi:10.1186/s12870-016-0771-y.

Wang W-H, Köhler B, Cao F-Q, Liu L-H. 2008. Molecular and physiological aspects of urea transport in higher plants. *Plant Science,* **175**: 467-477, doi:10.1016/j.plantsci.2008.05.018.

Wasternack C. 2007. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Annals of Botany,* **100**: 681-97, doi:10.1093/aob/mcm079.

Wasternack C, Hause B. 2013. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. *Annals of Botany,* **111**: 1021-58, doi:10.1093/aob/mct067.

Witte C-P. 2011. Urea metabolism in plants. *Plant Science,* **180**: 431-438, doi:10.1016/j.plantsci.2010.11.010.

Wolters H, Jürgens G. 2009. Survival of the flexible: hormonal growth control and adaptation in plant development. *Nature Reviews Genetics,* **10**: 305-317, doi:10.1038/nrg2558.

Wright PA. 1995. Nitrogen excretion: three end products, many physiological roles. *Journal of Experimental Biology,* **198**: 273-81.

7. Curriculum Vitae

Personal data

Name:	Ayufu Yilamujiang	
Date of birth:	17.09.1983	
Place of berth:	Kashgar	
Nationality:	China (Uyghur)	
Marital status:	married	
Email:	ayilamujiang@ice.mpg.de	ilhamayup@gmail.com

Scientific career and education

Since 10/2012	PhD student at the Max-Plank-Institute for Chemical ecology in Jena and Friedrich-schiller-University Jena Graduate Academy: International Max Planck Research School Jena (IMPRS): Exploration of ecological interactions with molecular and chemical techniques
11/2011-09/2012	Diploma thesis: The Identification of putative interacting protein partners of the calcium sensor protein, CML42, supervised by PD Dr. Axel Mithöfer (Max-Plank-Institute for Chemical ecology, Department of bioorganic chemistry) and Prof. Dr. Ralf Oelmüller (Friedrich-schiller-University Jena)
10/2008-09/2012	Diploma biochemistry/molecular biology, Friedrich-schiller-University Jena, Germany
03/2006-06/2006	Bachelor Thesis: Characterization of the factors changed in hypothalamic-pituitary-adrenal gland axis (HPA) in Abnormal Savda Syndrome carrier mice of Uighur traditional medicine (Institute for Biochemistry, Xinjiang Agricultural University, Urumchi, Xinjiang, China
09/2001-06/2006	Biotechnology (Bachelor of Science (B.Sc.) including 1 year of Chinese language study), Institute for Biochemistry, Xinjiang Agricultural University, China

Attended courses

Aug-Sep 2016	Introduction to Basic Statistics and R, speaker: Grit Kunert
Jan 2016	Adobe Photoshop & Illustrator, speaker: Dr. Nico Überschaar
Mar 2015	NMR course, speaker: Dr. Bernd Schneider and co-workers
Jul 2014	Metabolomics principles and fundamental techniques, speaker: Prof. Georg Pohnert and co-workers
Apr 2014	Professional Job Application for PhD Students, speaker: Barbara Hoffbauer, KEPOS
Apr 2014	Introduction to Microscopy
Feb-Mar 2014	Plant Secondary Metabolism, speaker: Prof. Jonathan Gershenzon, Dr. A. Hammerbacher, Dr. L. Wright
Jan 2014	Scientific Writing, speaker: Dr. Brian Cusack, ScienceCraft
Jul 2013	Molecular Evolution, speaker: Prof. David Heckel
Jul 2013	Fundamentals of Mass Spectrometry, speaker: Dr. A. Atthygalle, Dr. A. Svatos
Jun 2013	Fortbildungsveranstaltung nach Gentechnik- Sicherheitsverordnung

Publications

2017

Ayufu Yilamujiang, Anting Zhu, Rodrigo Ligabue-Braun, Stefan Bartram, Claus-Peter Witte, Rainer Hedrich, Mitsuyasu Hasabe, Caroline R. Schöner, Michael G. Schöner, Gerald Kerth, Célia R. Carlini, Axel Mithöfer (2017). Coprophagous features in carnivorous Nepenthes plants: a task for ureases. Submitted, Nature Ecology & Evolution

2016

Yilamujiang, A., Reichelt, M., Mithöfer, A. (2016). Slow food: insect prey and chitin induce phytohormone accumulation and gene expression in carnivorous Nepenthes plants. Annals of Botany, 118, 369-375. doi:10.1093/aob/mcw110.

2015

Buch, F., Kaman, W. E., Bikker, F. J., Yilamujiang, A., Mithöfer, A. (2015). Nepenthesin protease activity indicates digestive fluid dynamics in carnivorous Nepenthes plants. PLoS One, 10(3): e0118853. doi:10.1371/journal.pone.0118853.

2012

Yilamujiang, A., Vadassery, J., Boland, W., Mithöfer, A. (2012). Calmodulin-like proteins, CMLs: New players in plant defense regulation? Endocytobiosis and Cell Research, 22, 66-69.

Oral Presentations

Yilamujiang A. (2015). Insect prey induces gene expression and phytohormone accumulation in traps of carnivorous Nepenthes plants. Talk presented at Seminário Geral, Universidade Federal do Rio Grande do Sul, Porto Alegra, Porto Alegre, BR (invited speaker)

Yilamujiang A. (2015). Regulation of carnivory-related signaling in Nepenthes. Talk presented at 14th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

Poster Presentations

Yilamujiang A., Bartram S., Zhu A., Ligabue-Braun R., Schöner C., Carlini C., Witte C.-P., Mithöfer A. (2016). Coprophagous features in carnivorous Nepenthes plants: a role for ureases. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE

Yilamujiang A. (2016). Prey induced phytohormones and gene expression in carnivorous Nepenthes. Poster presented at 4th International Symposium on Plant Signaling & Behavior 2016, St. Petersburg, RU

Yilamujiang A. (2016). Prey induced phytohormones and gene expression in carnivorous Nepenthes. Poster presented at 15th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

Buch F., Yilamujiang A., Mithöfer A. (2014). Molecular regulation of carnivory in Nepenthes spec. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE

Buch F., Yilamujiang A., Mithöfer A. (2014). Molecular regulation of carnivory in Nepenthes spec. Poster presented at SAB Meeting 2014, MPI for Chemical Ecology, Jena, DE

Yilamujiang A., Mithöfer A., Reichelt M., Buch F. (2014). Towards promoter analysis for pathogeneses-related (PR) protein genes in carnivorous plants. Poster presented at 13th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

Yilamujiang A., Buch F., Reichelt M., Mithöfer A. (2013). A role of phytohormones in carnivory in pitcher plants. Poster presented at Botanikertagung 2013, Deutsche Botanische Gesellschaft, Tübingen, DE

Yilamujiang A. (2013). A Role for Phytohormones in carnivorous Plants? Poster presented at 9th Plant Science Student Conference - PSSC 2013, Leibniz-Institut für Pflanzenbiochemie, Halle (Saale), DE

Yilamujiang A. (2013). A role for phytohormones in carnivorous plants? Poster presented at 12th IMPRS Symposium, MPI for Chemical Ecology, Jena, DE

8. Acknowledgement

I would like to express my great appreciation for all the people who supported me on the way of my like.

First of all special thanks goes to PD Dr. Axel Mithöfer, who not only supervised my work but also provided me with the confidence and liberty to follow all my interests and ideas. I also like to thank for him to allowing me to work in his group and for the friendship. I would like to thank Prof Dr. Wilhelm Boland for giving me the opportunity to work in the department. I thank him and the Max Planck Society for continuous support. I thank my Co-supervisor Prof. Dr. Ralf Oelmüller. I would like to express my appreciation to Prof. Célia R. Carlini for her contribution for the development of the urease project. I am pleased that PD Dr. Klaus Appenroth and Prof Dr. Irene Lichtscheidl accepted to be a reviewer for my thesis.

I also would like to thank Dr. Jyothilakshmi Vadassery and Dr. Sandra Scholz who supervised me in the lab at the first step of my scientific career in the institute. Thank you very much for supporting me till now. I would really like to thank for all the former and current members of the plant defense group, who helped and accepted me from their heart. At the same time, I would like to thank all the members of bioorganic chemistry department for their friendship. I thank for all of you for your contribution for creating fruitful working atmosphere. I want to express my additional thanks for Sandra, Shraddha, Abith, Stefan for your really helpful support in the last few days. The people not only in the department but also in the whole institute influenced my scientific approach and way of thinking during innumerable discussions. Many thanks for all of you. I thank Birgit Arnold and the whole greenhouse team at the MPI for chemical ecology. Members of the IT department and library, thank you Martin, Linda.

I freue mich, dass ich in Deutchland studiert habe und konnte.

Danke Deutschland für die tollen Möglichkeiten!

I would like to really thank for my wife, Patigül and to my son, Eldin. You made my life more meaningful. You are contributing lot of values and lot of fun to my life.

I am grateful for the people enriched my privet life by giving me friendship:

Thank you Robby, Cora, Max, Anne!

I know that there are lots of people, who were really important in my scientific and private life, which I have to mention, but not be mentioned by their names in this acknowledgement. However, I have to say that I will never forget the people who really helped me. I try all my best to give them back, more than that, what they have given me.

Very importantly, my whole family supported me a lot. Now, I want to use this opportunity to express my appreciation by using my mother language (Uyghur language):

Dada (Ayup Qurban), Anna (Asigul Nasirdin), ukullurum Imamjan, Ibrayimjan, Ilmigul baharliq uruq tuqqanlar, silarning uzun yildin biri mini qollap kiliwatqininglargha koptin kop rexmet. Silerning qollushunglar bilen ahiri kashqardin kilip germaniyening MAX-PLANK INSTITUTidin tabi-penler docturluq unwanini elish aldida turuptiman. Buning hammisida silerning zor tohpanglar bar älkwatta. Muttallap aka wa bashqa uruq tuqqanlar silergimu kop rehmet!!!!!!!!!!!!!!!

Ayufu Yilamujiang(Ilham Ayup)

02.02.2017 Jena Germany

9. Eigenständigkeitserklärung

Ich erkläre hiermit, dass mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist.

Ich habe die vorliegende Dissertation selbständig verfasst und alle Hilfsmittel und Quellen in der Arbeit angegeben.

Alle Personen, die an den Manuskripten durch Experimente, Auswertung und Schreiben mitgewirkt haben, sind im Kapitel „*Manuscript Overview*“ mit ihrem jeweiligen Anteil aufgelistet.

Ich habe weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte unmittelbar oder mittelbar geldwerte Leistungen von mir für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorliegenden Arbeit stehen.

Ich habe die Dissertation noch nicht als Prüfungsarbeit zu einer staatlichen oder anderen wissenschaftlichen Prüfung eingereicht. Ferner habe ich auch nicht versucht, die gleiche, eine in wesentlichen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation einzureichen.

Ayufu Yilamujiang